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(54) **METHODS AND COMPOSITIONS FOR EVALUATING GENETIC MARKERS**

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None
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(57) **ABSTRACT**

Aspects of the invention relates to methods and compositions that are useful to reduce bias and increase the reproducibility of multiplex analysis of genetic loci. In some configurations, predetermined preparative steps and/or nucleic acid sequence analysis techniques are used in multiplex analyses for a plurality of genetic loci in a plurality of samples.

14 Claims, 42 Drawing Sheets

Specification includes a Sequence Listing.

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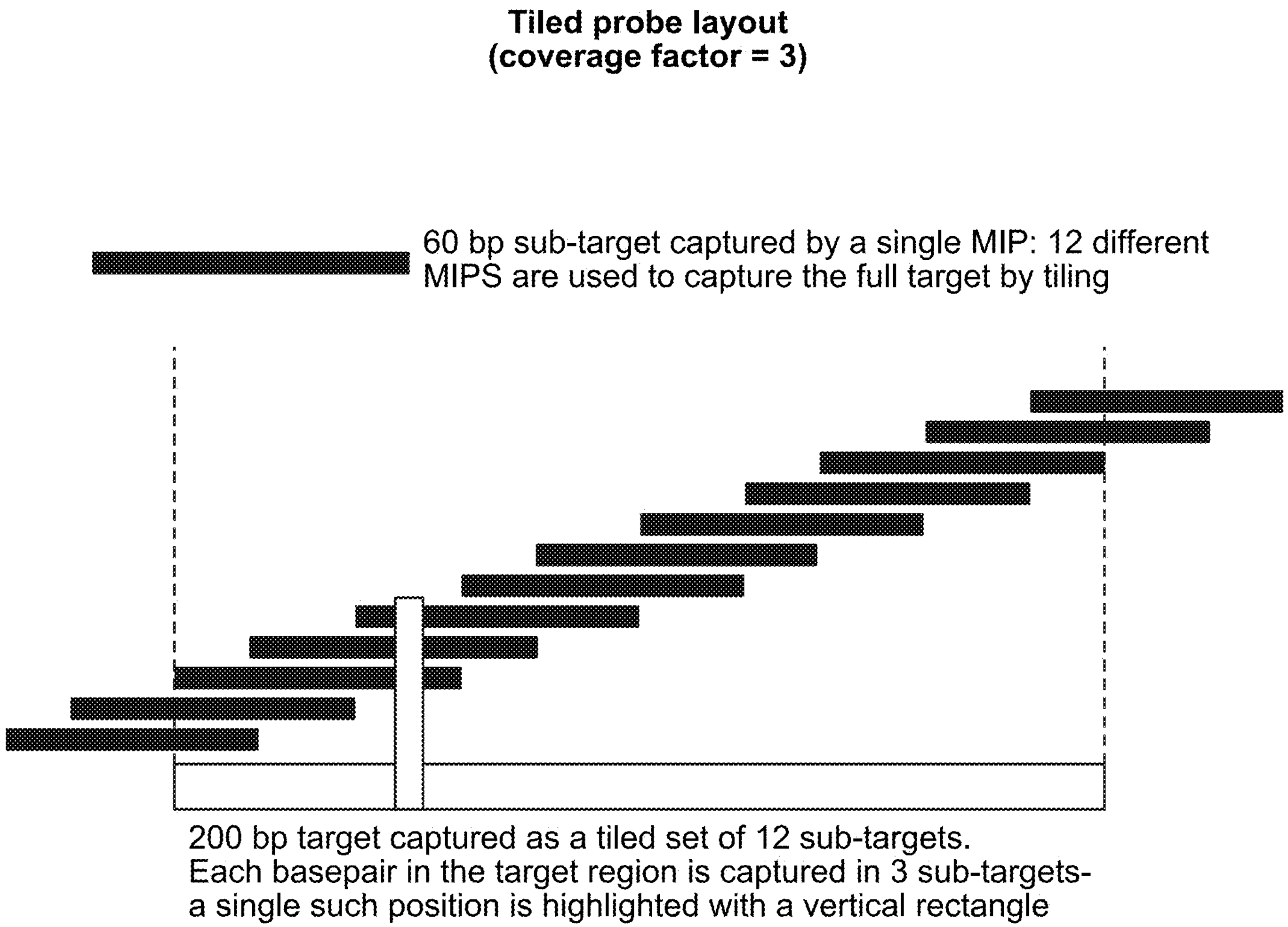
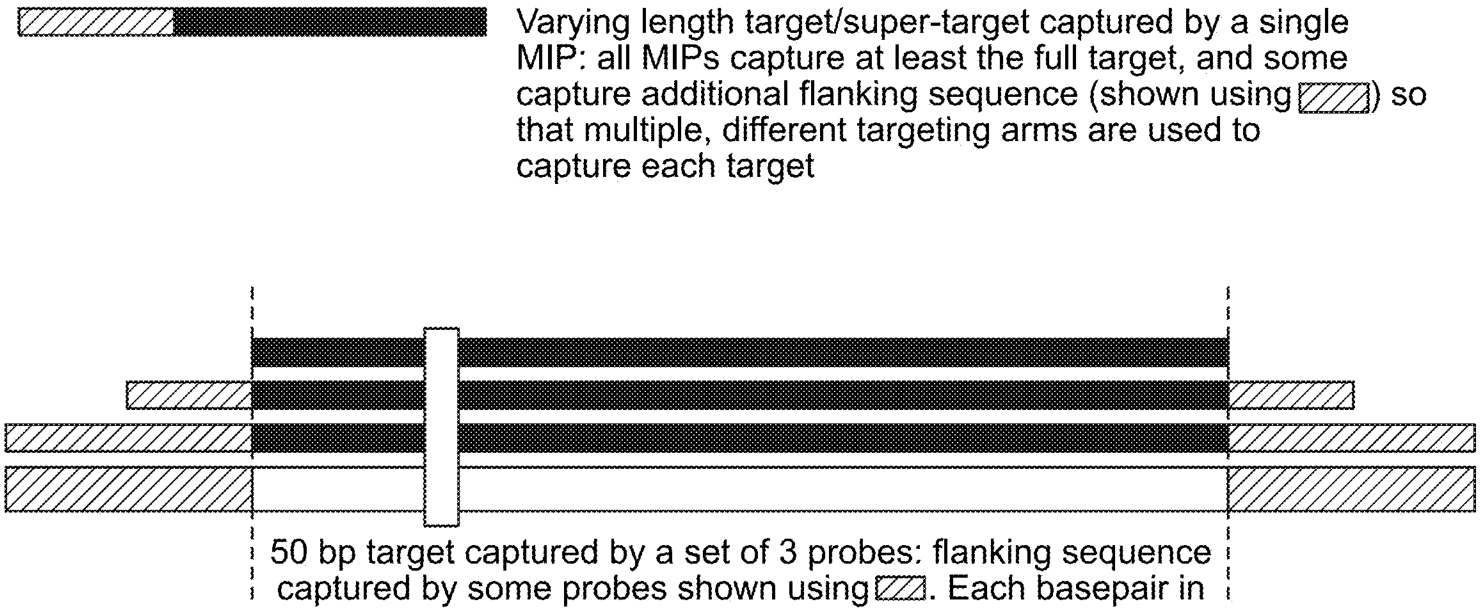

Tiled probe layout
(coverage factor = 3)
60 bp sub-target captured by a single MIP: 12 different MIPS are used to capture the full target by tiling
200 bp target captured as a tiled set of 12 sub-targets.
Each basepair in the target region is captured in 3 sub-targets- a single such position is highlighted with a vertical rectangle

FIG. 1

Staggered probe layout
(coverage factor = 3)

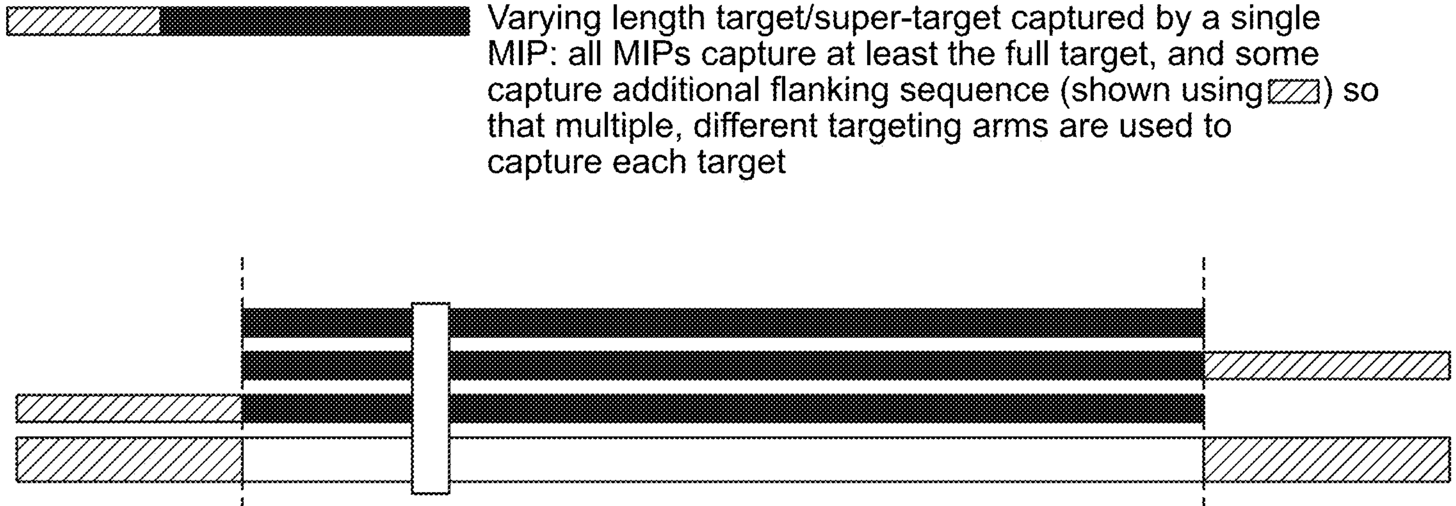


Varying length target/super-target captured by a single MIP: all MIPs capture at least the full target, and some capture additional flanking sequence (shown using ▨) so that multiple, different targeting arms are used to capture each target

50 bp target captured by a set of 3 probes: flanking sequence captured by some probes shown using ▨. Each basepair in the target region is captured in 3 sub-targets - a single such position is highlighted with a vertical rectangle

FIG. 2

Alternating staggered probe layout
(coverage factor = 3)



Varying length target/super-target captured by a single MIP: all MIPs capture at least the full target, and some capture additional flanking sequence (shown using ▨) so that multiple, different targeting arms are used to capture each target

50 bp target captured by a set of 3 probes: flanking sequence captured by some probes shown using ▨. Each basepair in the target region is captured in 3 sub-targets - a single such position is highlighted with a vertical rectangle

FIG. 3

FIGURE 4

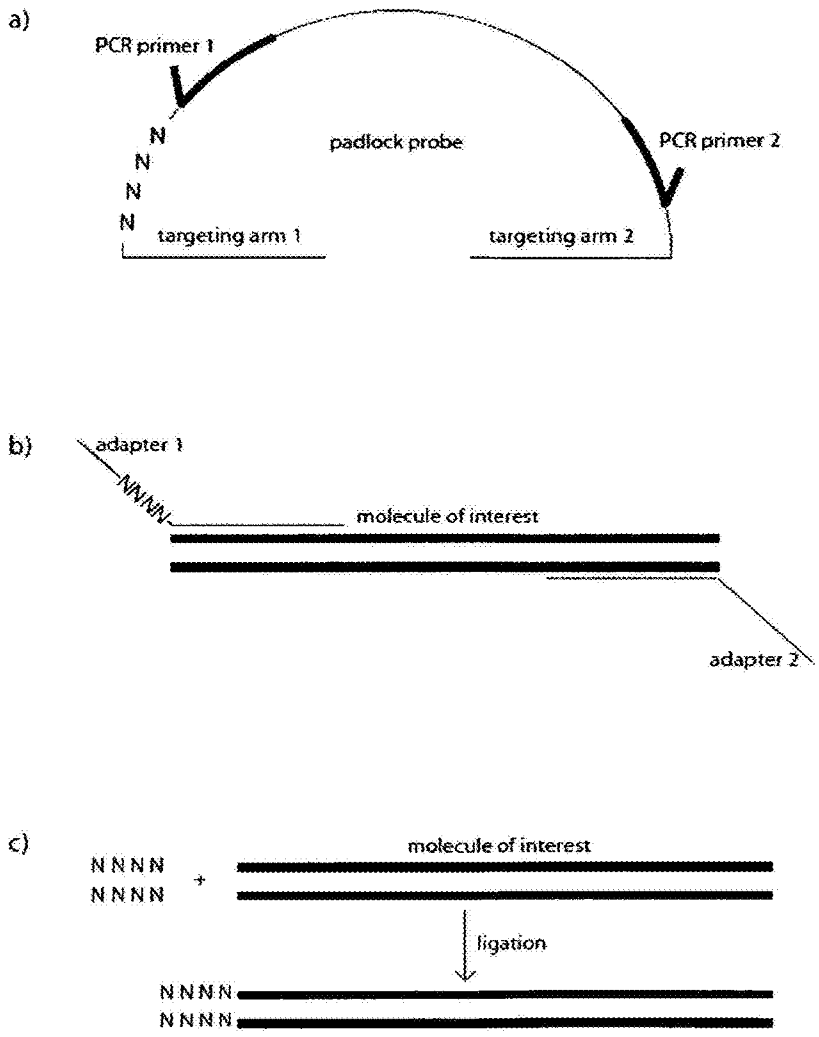

a)
PCR primer 1
N
N
N
N
padlock probe
PCR primer 2
targeting arm 1
targeting arm 2
b)
adapter 1
NNNN
molecule of interest
adapter 2
c)
molecule of interest
N N N N
N N N N
+
ligation
N N N N
N N N N

FIGURE 5

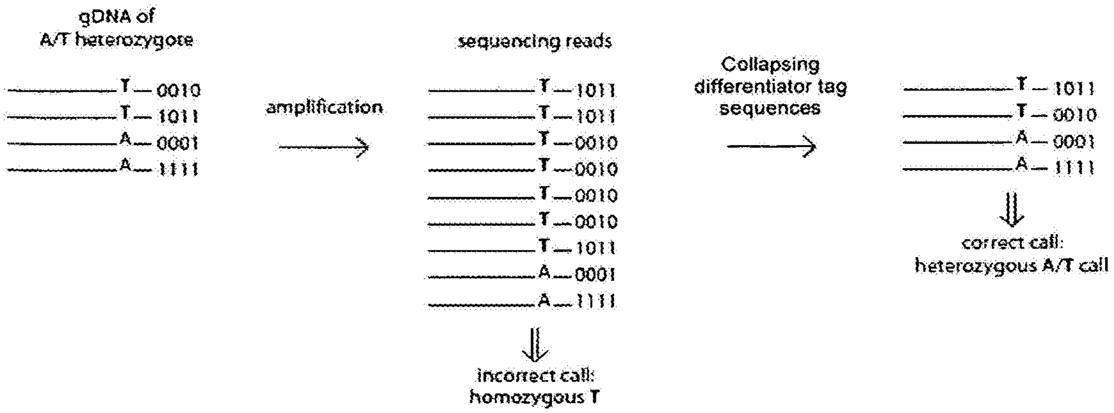
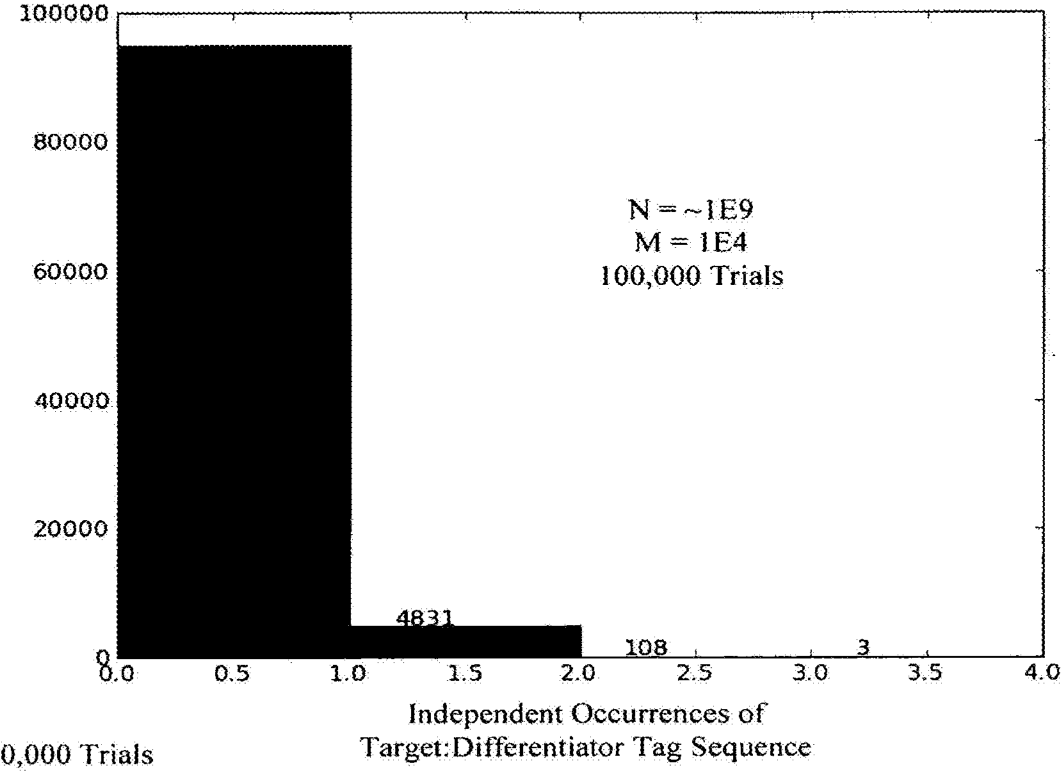

gDNA of
A/T heterozygote
T — 0010
T — 1011
A — 0001
A — 1111
amplification
sequencing reads
T — 1011
T — 1011
T — 0010
T — 0010
T — 0010
T — 0010
T — 1011
A — 0001
A — 1111
incorrect call:
homozygous T
Collapsing
differentiator tag
sequences
T — 1011
T — 0010
A — 0001
A — 1111
correct call:
heterozygous A/T call

FIGURE 6



100,000 Trials

FIGURE 7

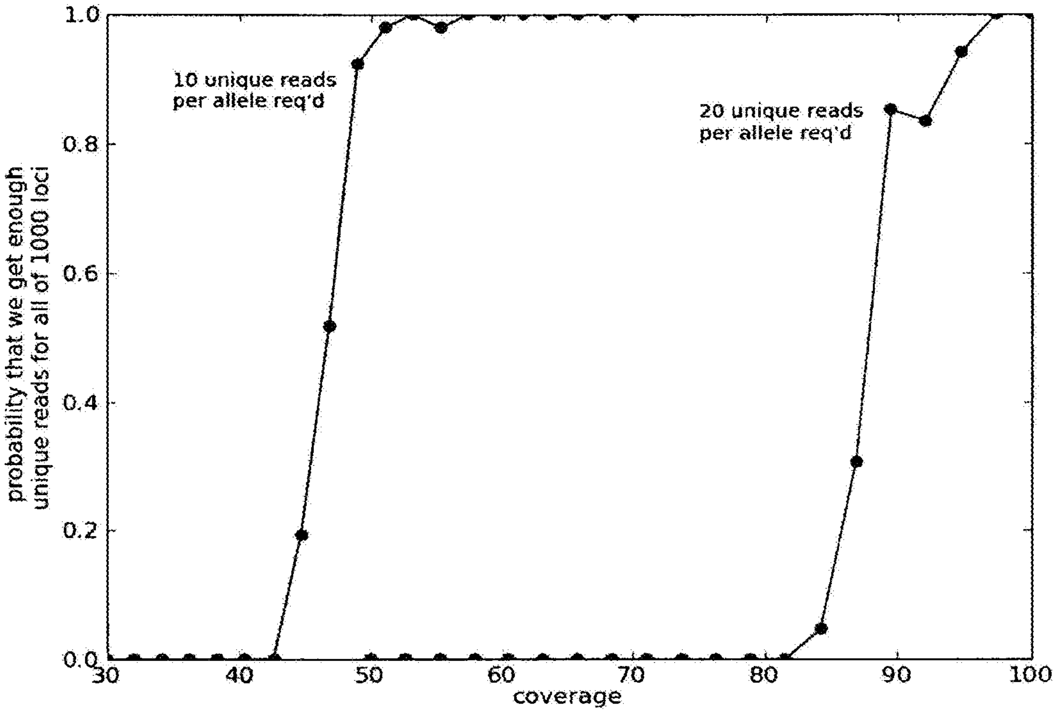

1.0
0.8
0.6
0.4
0.2
0.0
probability that we get enough unique reads for all of 1000 loci
30
40
50
60
70
80
90
100
coverage
10 unique reads per allele req'd
20 unique reads per allele req'd

Figure 8

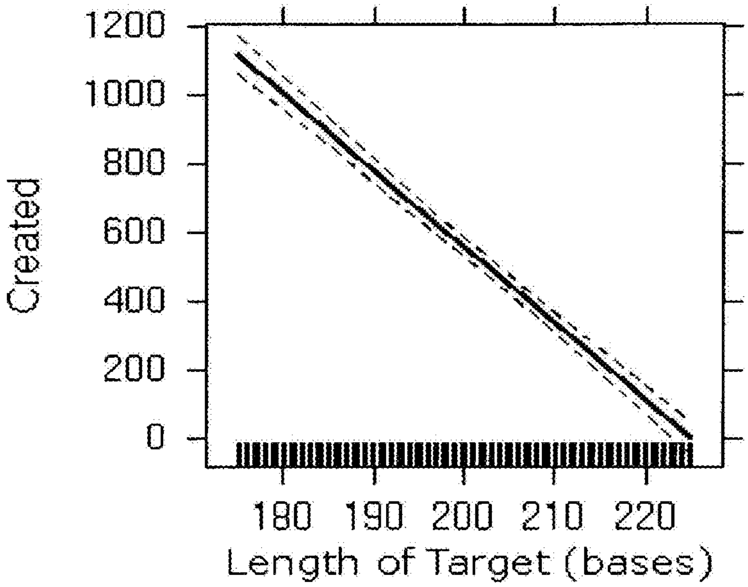

1200
1000
800
600
400
200
0
Created
180
190
200
210
220
Length of Target (bases)

Figure 9

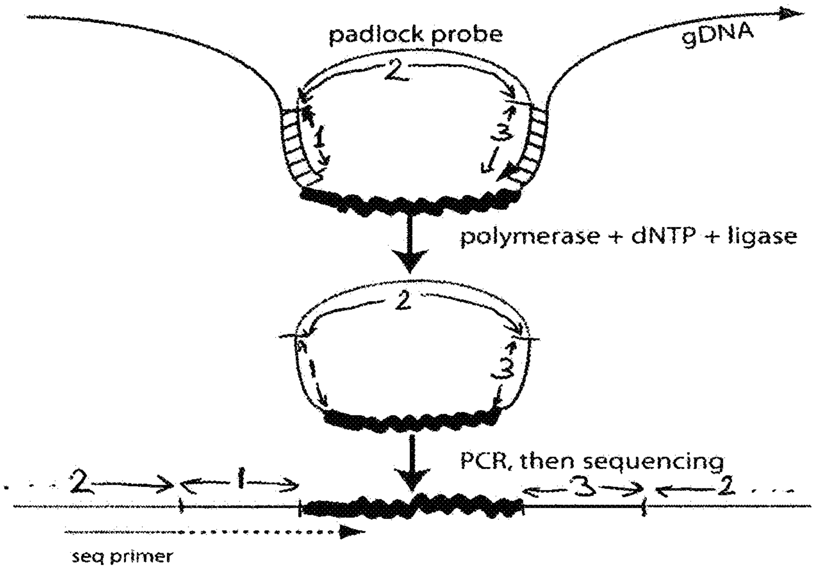

padlock probe
gDNA
2
1
3
polymerase + dNTP + ligase
2
1
3
PCR, then sequencing
. . . 2
1
3
2 . . .
seq primer

Figure 10

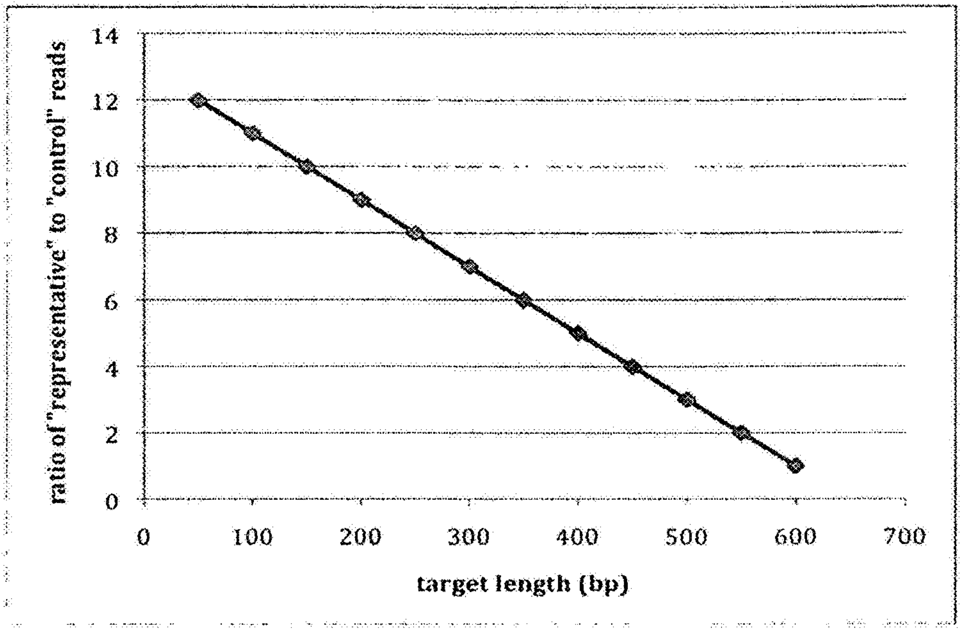

ratio of "representative" to "control" reads
14
12
10
8
6
4
2
0
0
100
200
300
400
500
600
700
target length (bp)

FIGURE 11A

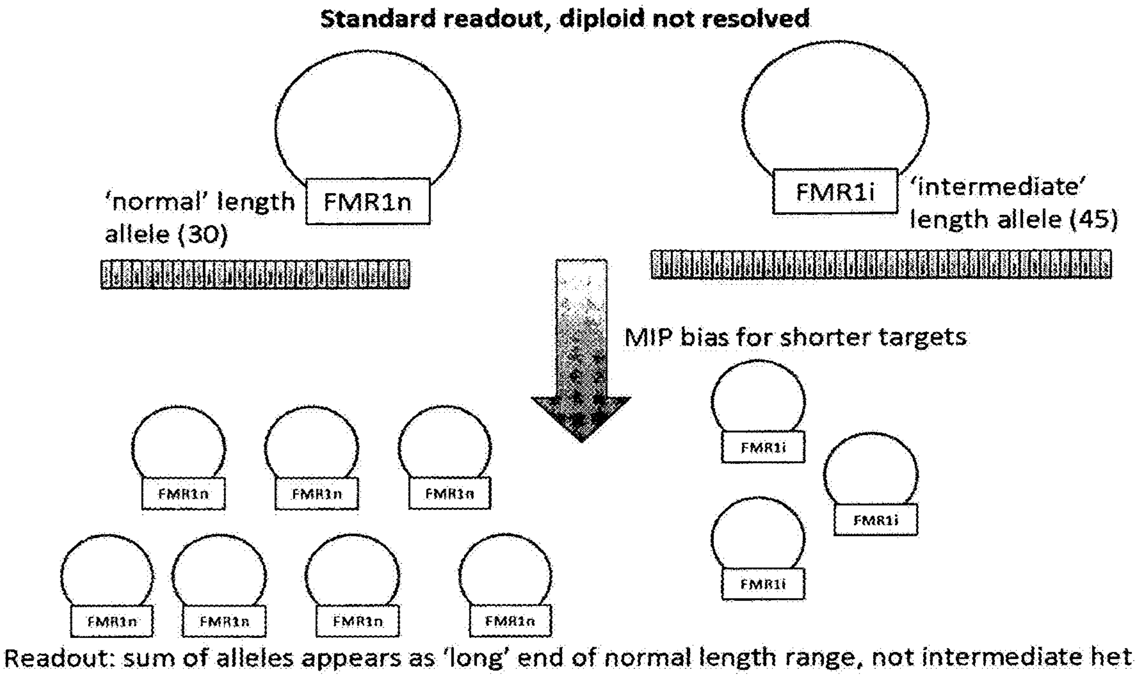

Standard readout, diploid not resolved
'normal' length allele (30)
FMR1n
FMR1i
'intermediate' length allele (45)
MIP bias for shorter targets
FMR1n
FMR1n
FMR1n
FMR1i
FMR1i
FMR1i
FMR1n
FMR1n
FMR1n
FMR1n
Readout: sum of alleles appears as 'long' end of normal length range, not intermediate het

FIGURE 11B

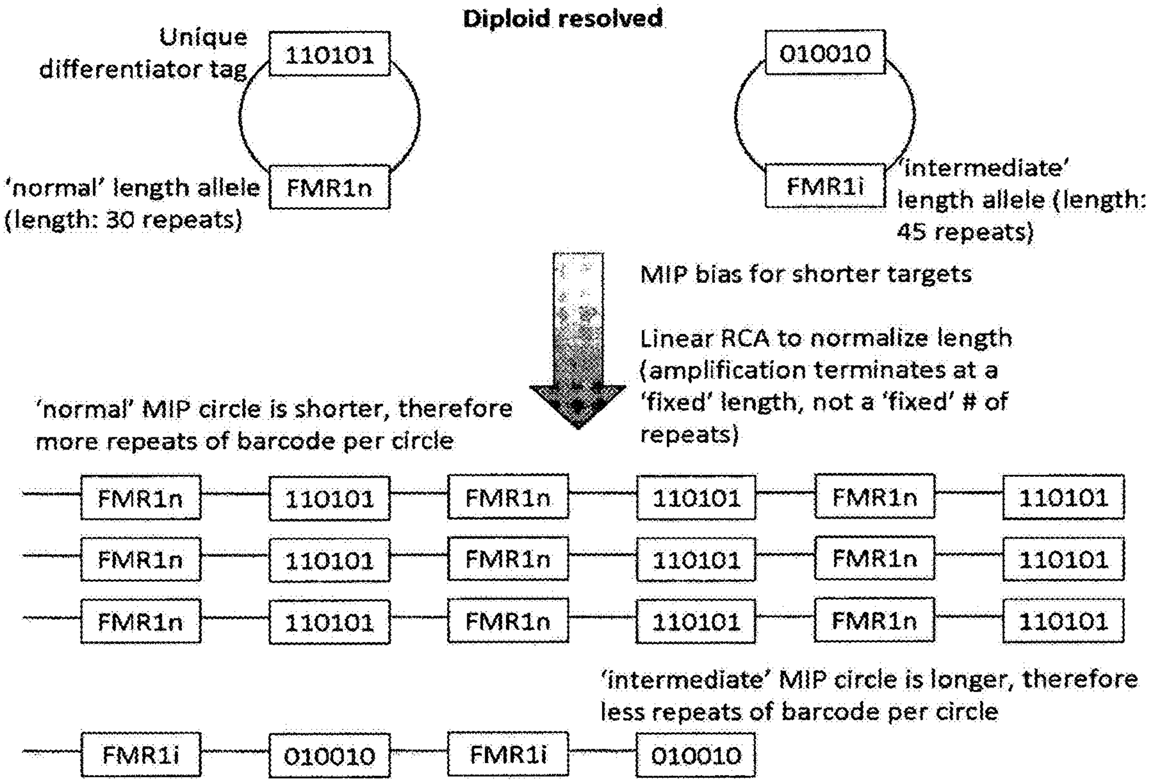

Diploid resolved
Unique differentiator tag
110101
FMR1n
'normal' length allele (length: 30 repeats)
010010
FMR1i
'intermediate' length allele (length: 45 repeats)
MIP bias for shorter targets
Linear RCA to normalize length (amplification terminates at a 'fixed' length, not a 'fixed' # of repeats)
'normal' MIP circle is shorter, therefore more repeats of barcode per circle
FMR1n
110101
FMR1n
110101
FMR1n
110101
FMR1n
110101
FMR1n
110101
FMR1n
110101
FMR1n
110101
FMR1n
110101
FMR1n
110101
'intermediate' MIP circle is longer, therefore less repeats of barcode per circle
FMR1i
010010
FMR1i
010010

FIGURE 11C

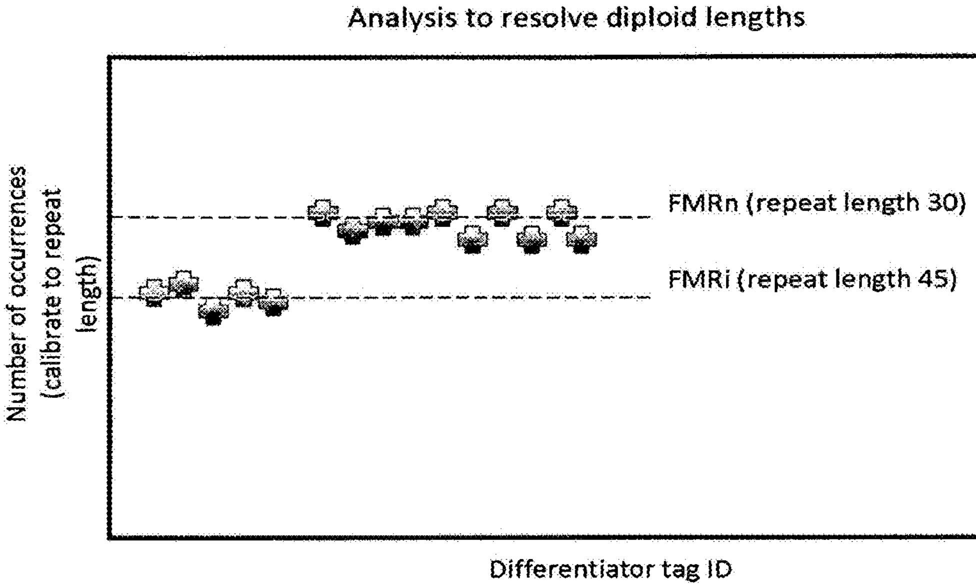

Analysis to resolve diploid lengths
Number of occurrences (calibrate to repeat length)
FMRn (repeat length 30)
FMRi (repeat length 45)
Differentiator tag ID

FIGURE 12

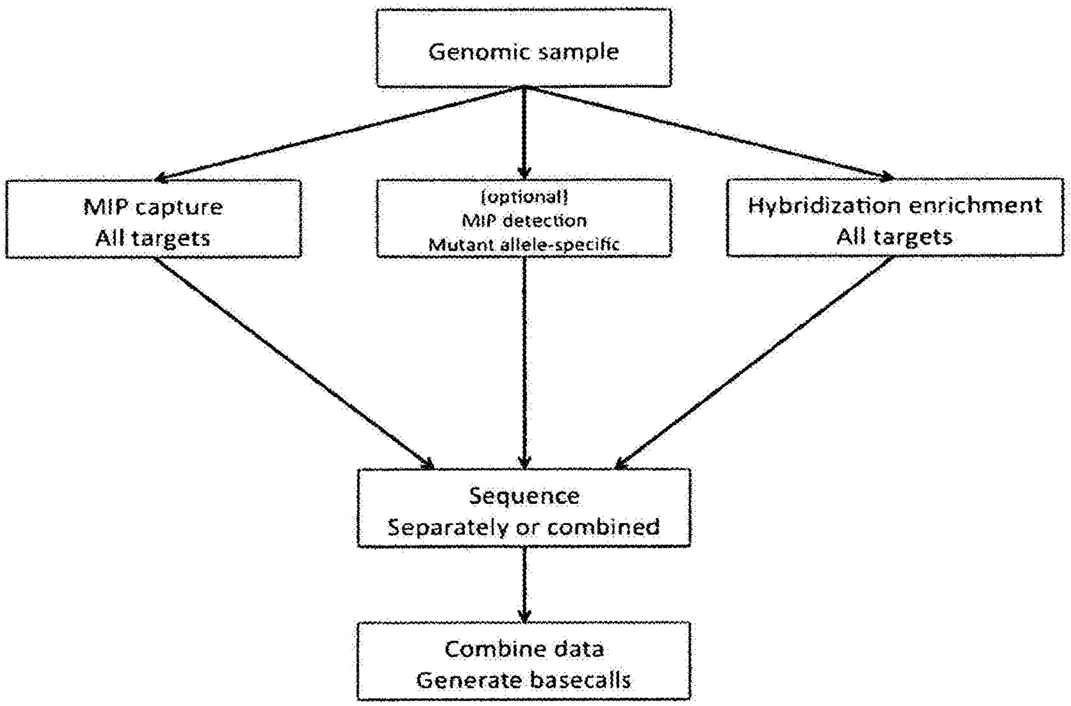

Genomic sample
MIP capture
All targets
(optional)
MIP detection
Mutant allele-specific
Hybridization enrichment
All targets
Sequence
Separately or combined
Combine data
Generate basecalls

FIGURE 13

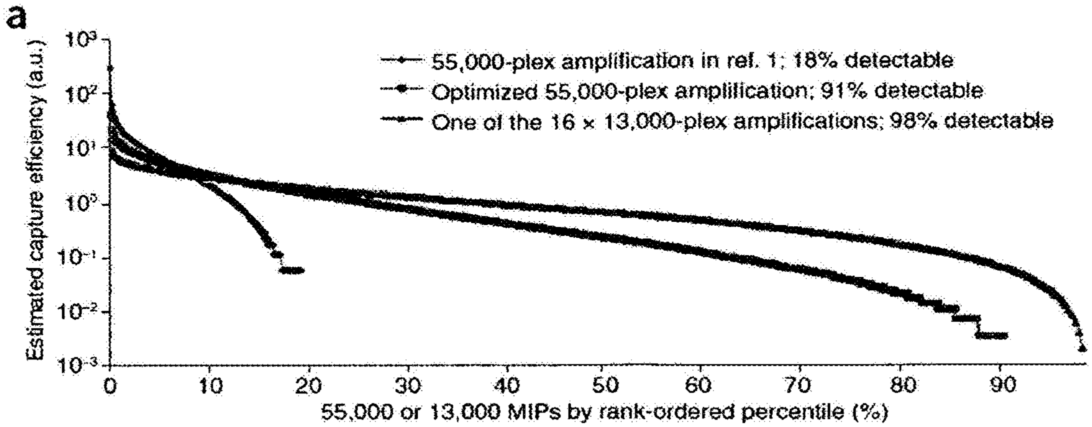

a
Estimated capture efficiency (a.u.)
10³
10²
10¹
10⁰
10⁻¹
10⁻²
10⁻³
0
10
20
30
40
50
60
70
80
90
55,000 or 13,000 MIPs by rank-ordered percentile (%)
55,000-plex amplification in ref. 1; 18% detectable
Optimized 55,000-plex amplification; 91% detectable
One of the 16 × 13,000-plex amplifications; 98% detectable

FIGURE 14

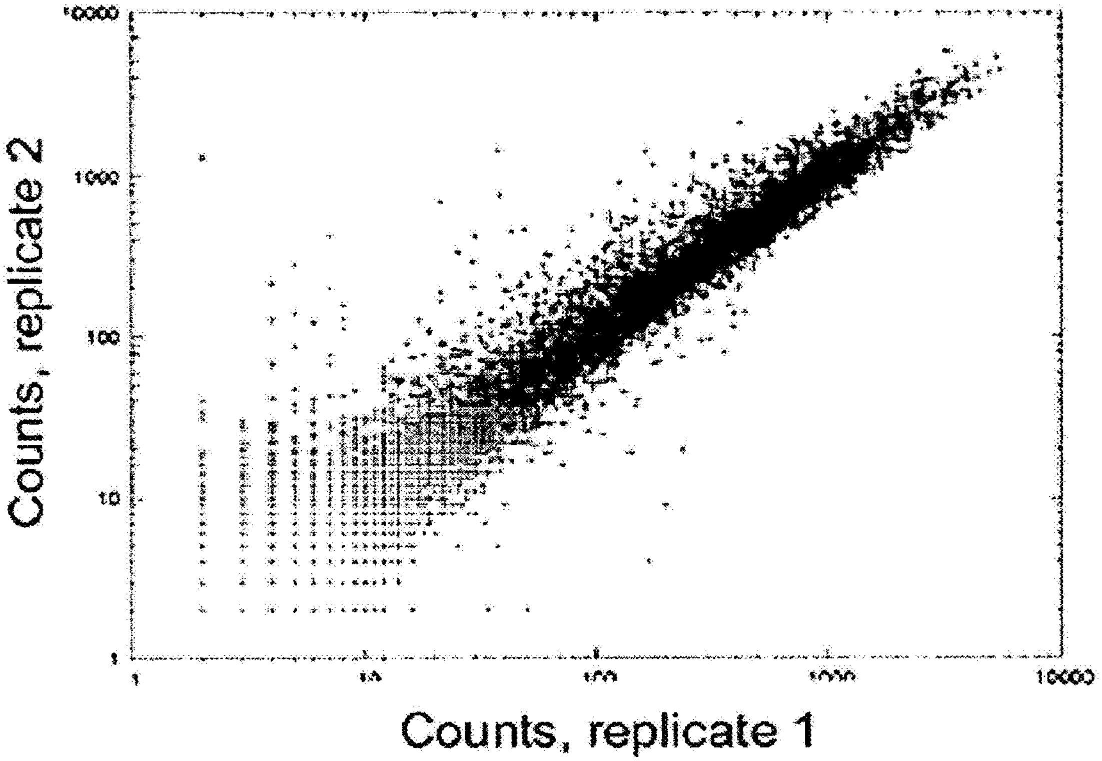

10000
1000
100
10
1
1
10
100
1000
10000
Counts, replicate 2
Counts, replicate 1

FIG. 15A

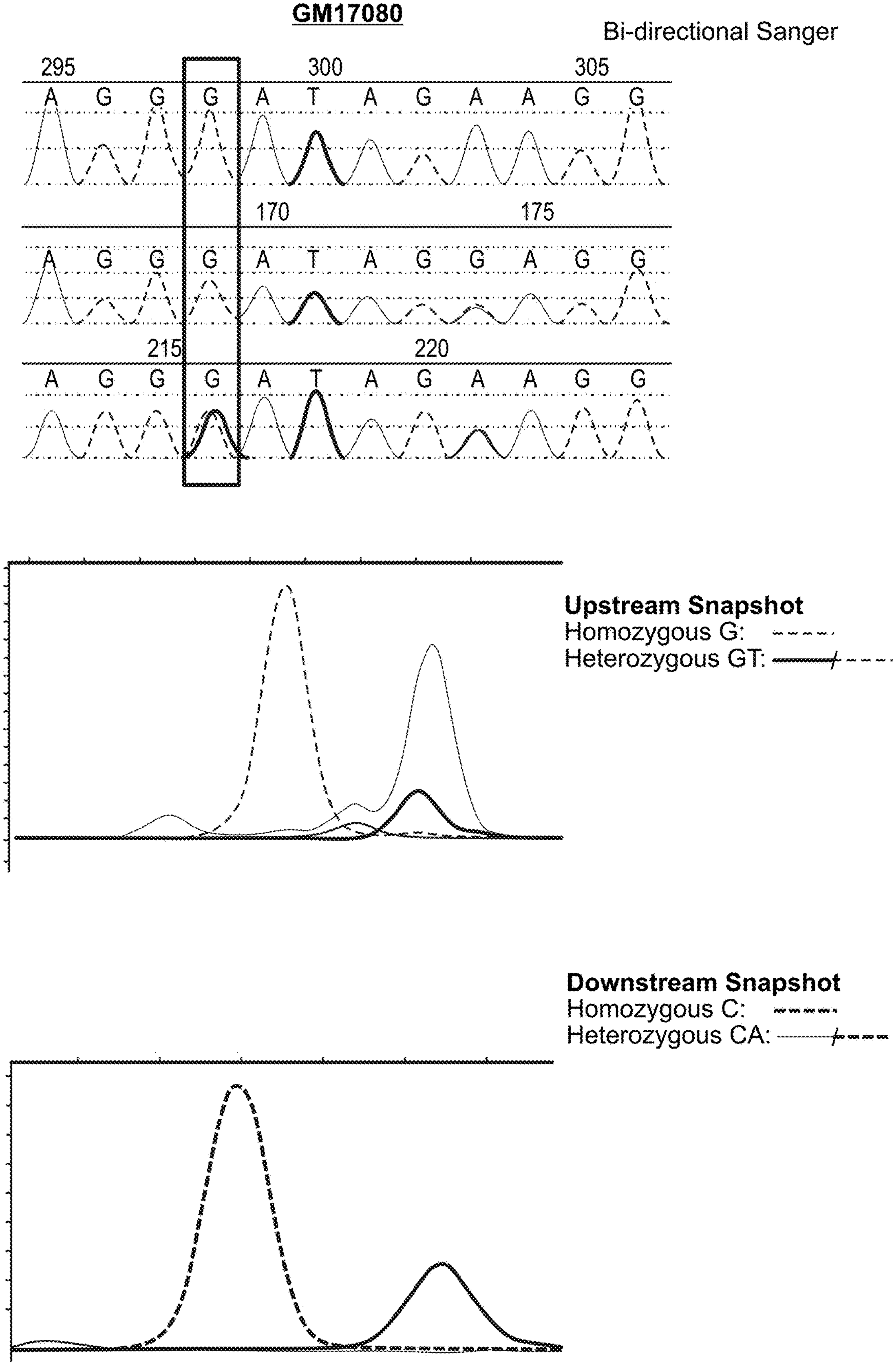
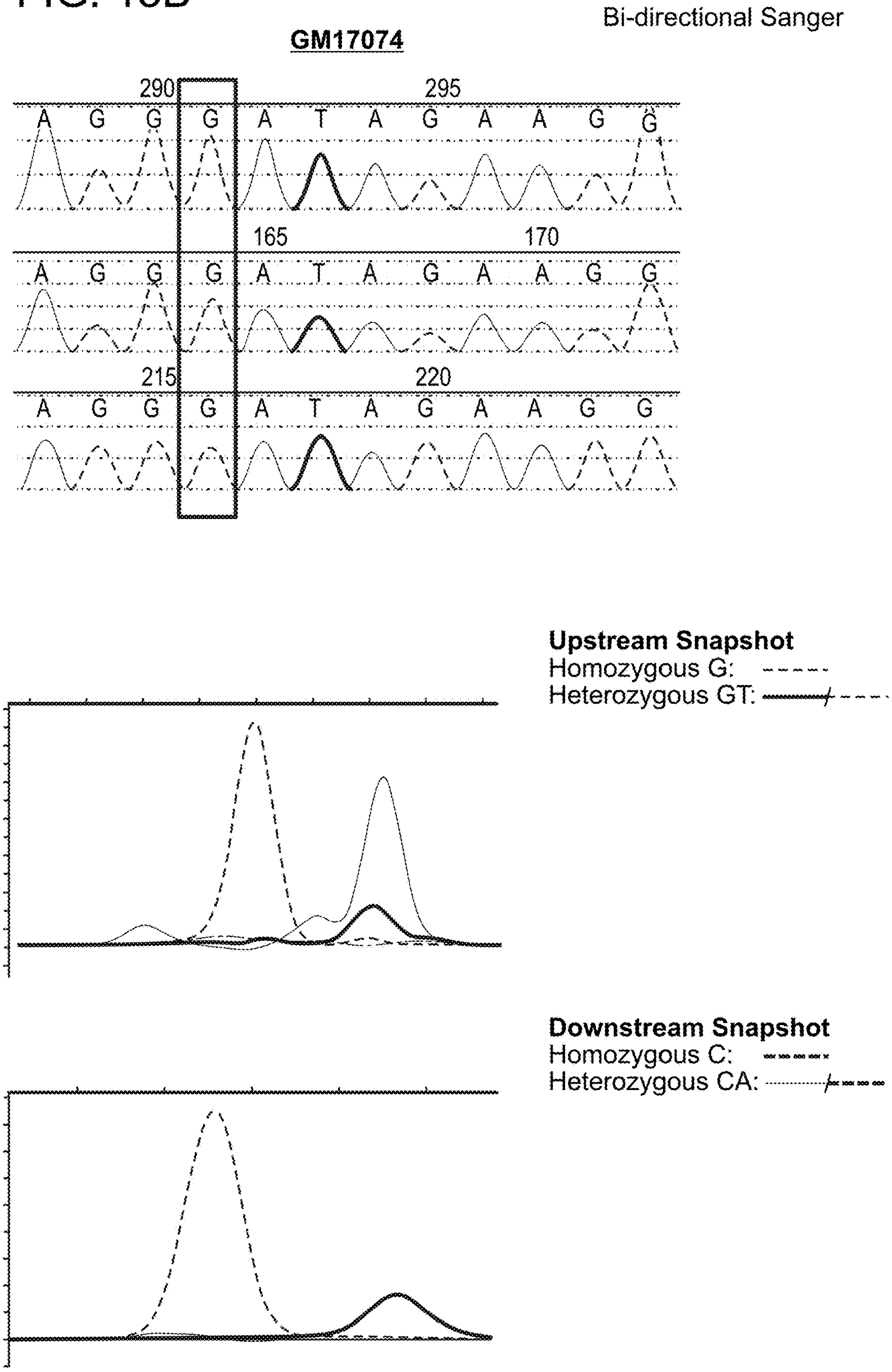

GM17080
Bi-directional Sanger
295
300
305
A G G G A T A G A A G G
170
175
A G G G A T A G G A G G
215
220
A G G G A T A G A A G G
Upstream Snapshot
Homozygous G: ----
Heterozygous GT: ——/----
Downstream Snapshot
Homozygous C: ----
Heterozygous CA: ——/----

FIG. 15B


Bi-directional Sanger
GM17074
290
295
A G G G A T A G A A G G
165
170
A G G G A T A G A A G G
215
220
A G G G A T A G A A G G
Upstream Snapshot
Homozygous G:
Heterozygous GT:
Downstream Snapshot
Homozygous C:
Heterozygous CA:

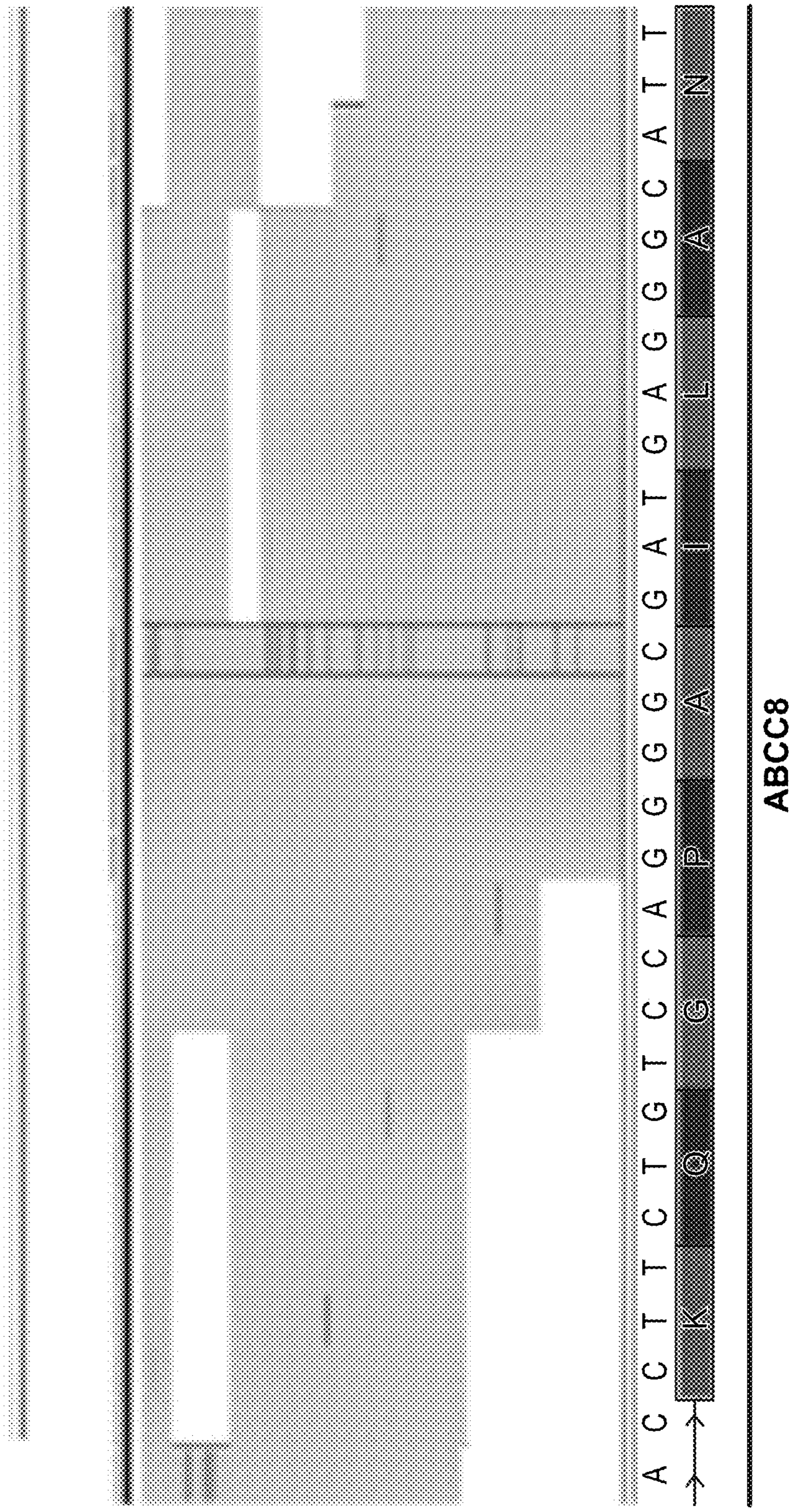


FIG. 16A

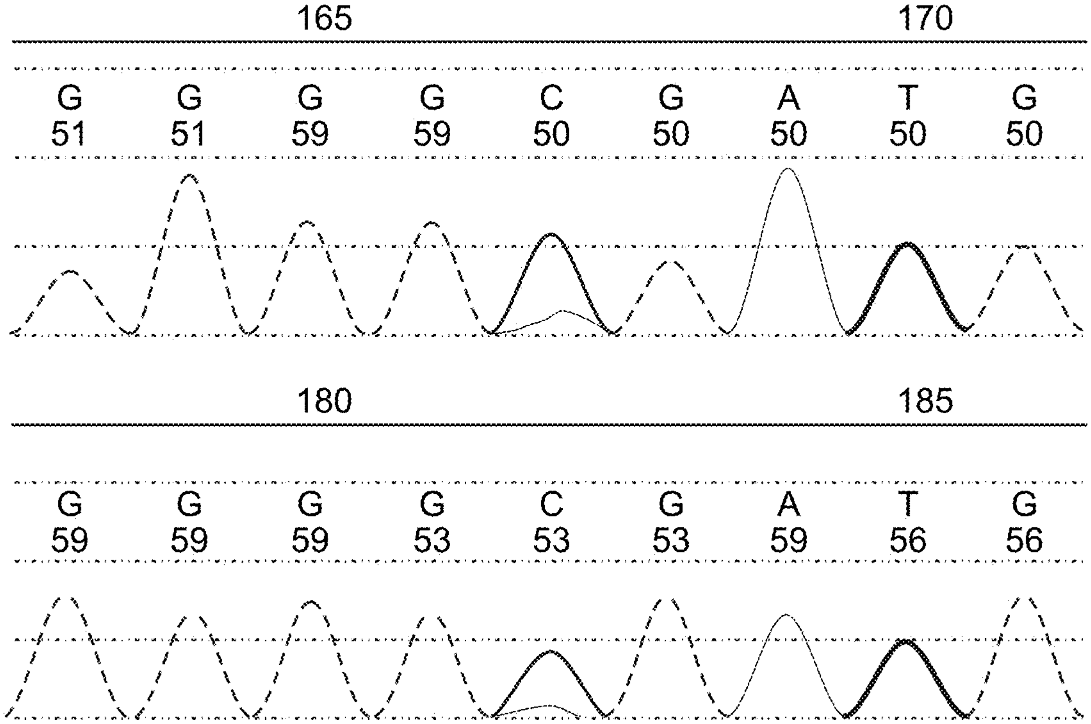

165
170
G
51
G
51
G
59
G
59
C
50
G
50
A
50
T
50
G
50
180
185
G
59
G
59
G
59
G
53
C
53
G
53
A
59
T
56
G
56

FIG. 16B

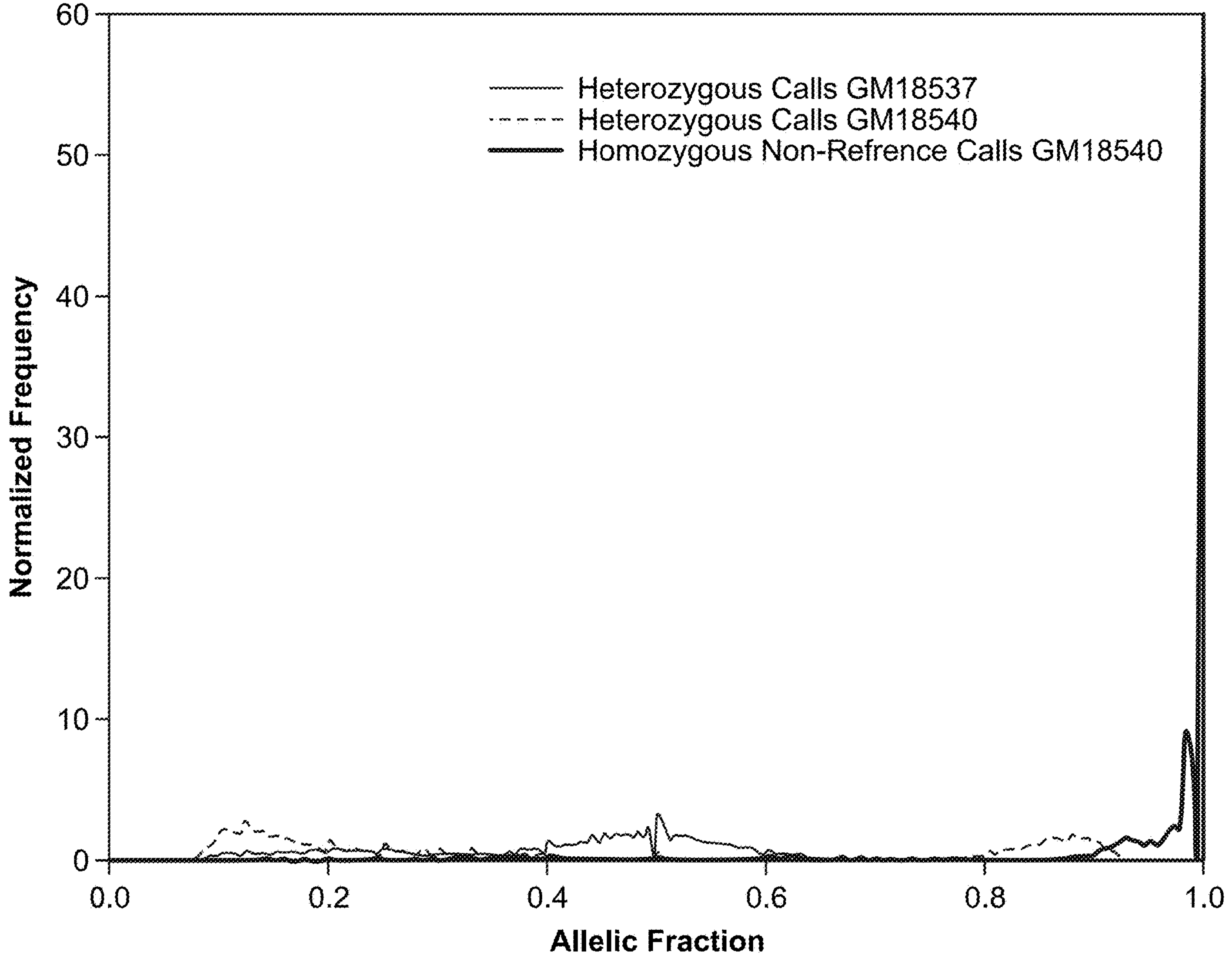
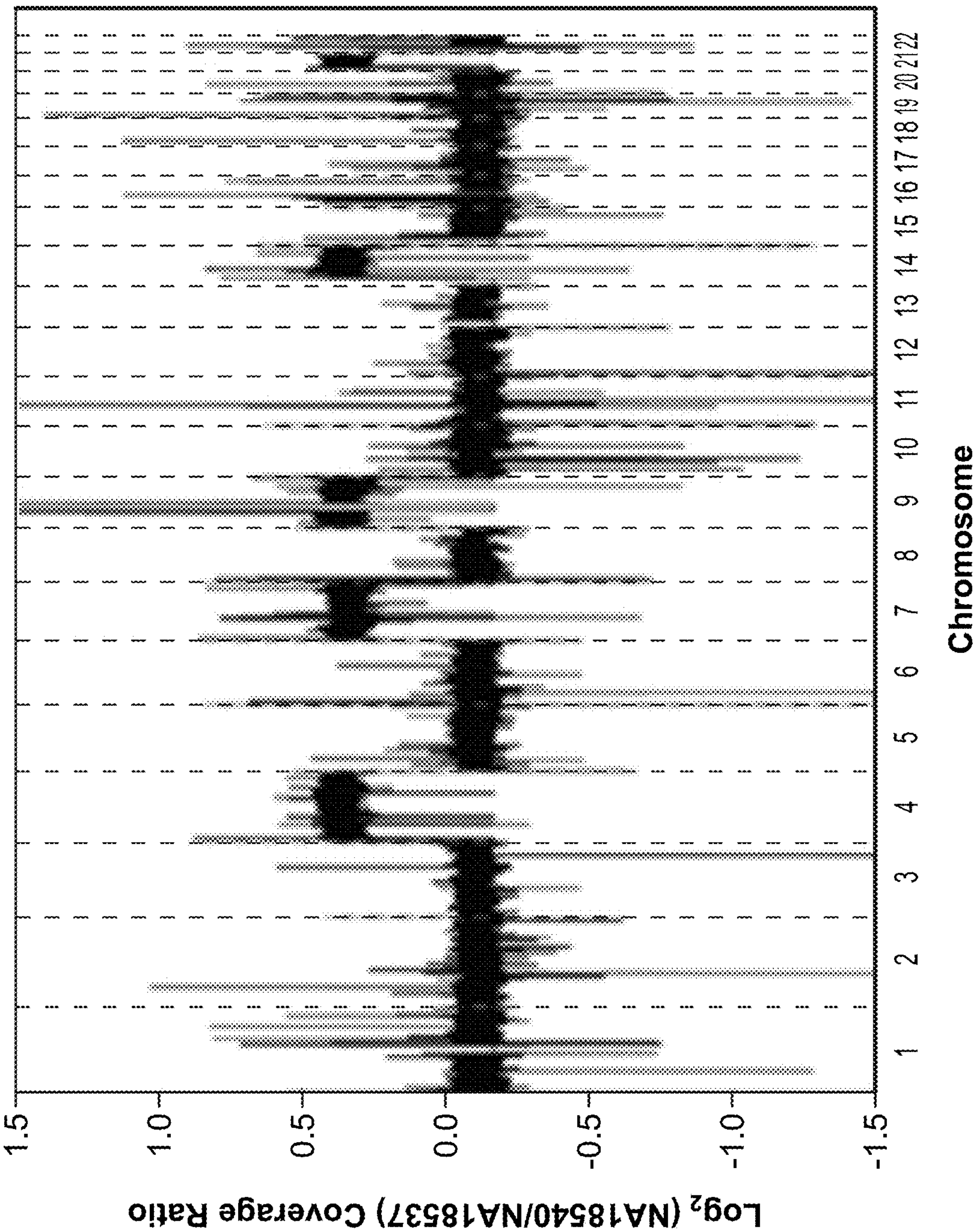

Heterozygous Calls GM18537
Heterozygous Calls GM18540
Homozygous Non-Refrence Calls GM18540
Normalized Frequency
60
50
40
30
20
10
0
0.0
0.2
0.4
0.6
0.8
1.0
Allelic Fraction

FIG. 16C

FIG. 16D


Log$_2$ (NA18540/NA18537) Coverage Ratio
1.5
1.0
0.5
0.0
-0.5
-1.0
-1.5
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
Chromosome

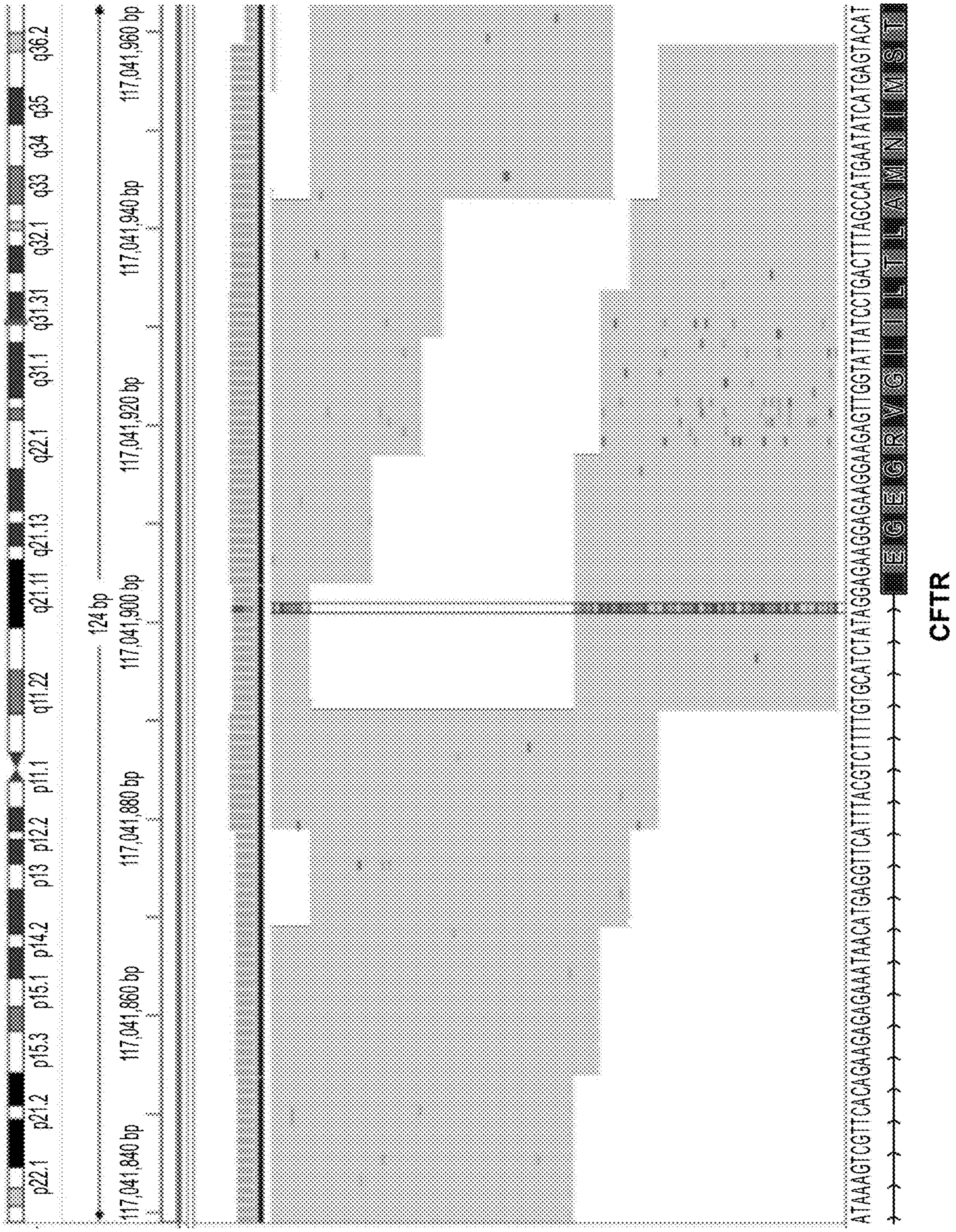

p22.1
p21.2
p15.3
p15.1
p14.2
p13
p12.2
p11.1
q11.22
q21.11
q21.13
q22.1
q31.1
q31.31
q32.1
q33
q34
q35
q36.2
124 bp
117,041,840 bp
117,041,860 bp
117,041,880 bp
117,041,900 bp
117,041,920 bp
117,041,940 bp
117,041,960 bp
ATAAAGTCGTTCACAGAAGAGAGAATAACATGAGGTTCATTTACGTCTTTTGTGCATCTATAGGAGAGGAGAGGAAGAGTTGGTATTATCCTGACTTAGCCATGAATATCATGAGTACAT
E G E G R V G I I L T L A M N I M S T
CFTR

FIG. 17A

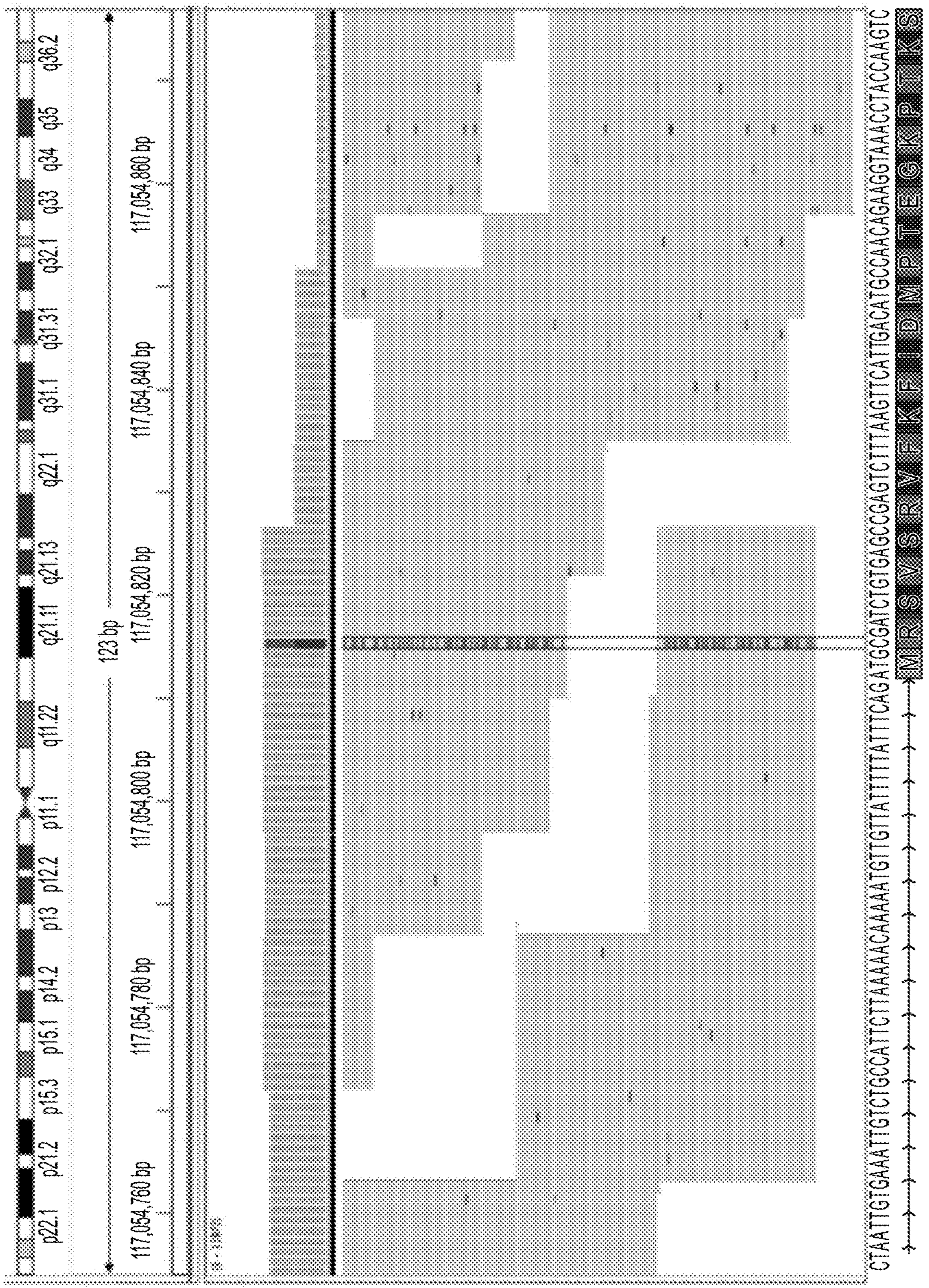

p22.1
p21.2
p15.3
p15.1
p14.2
p13
p12.2
p11.1
q11.22
q21.11
q21.13
q22.1
q31.1
q31.31
q32.1
q33
q34
q35
q36.2
123 bp
117,054,760 bp
117,054,780 bp
117,054,800 bp
117,054,820 bp
117,054,840 bp
117,054,860 bp
CTAATTGTGAAATTGTCTGCCATTCTTAAAAACAAAAATGTTGTTATTTTTATTTCAGATGCGATCTGTGAGCCGAGTCTTTAAGTTCATTGACATGCCAACAGAAGGTAAACCTACCAAGTC
M R S V S R V F K F I D M P T E G K P T K S
CFTR

FIG. 17B

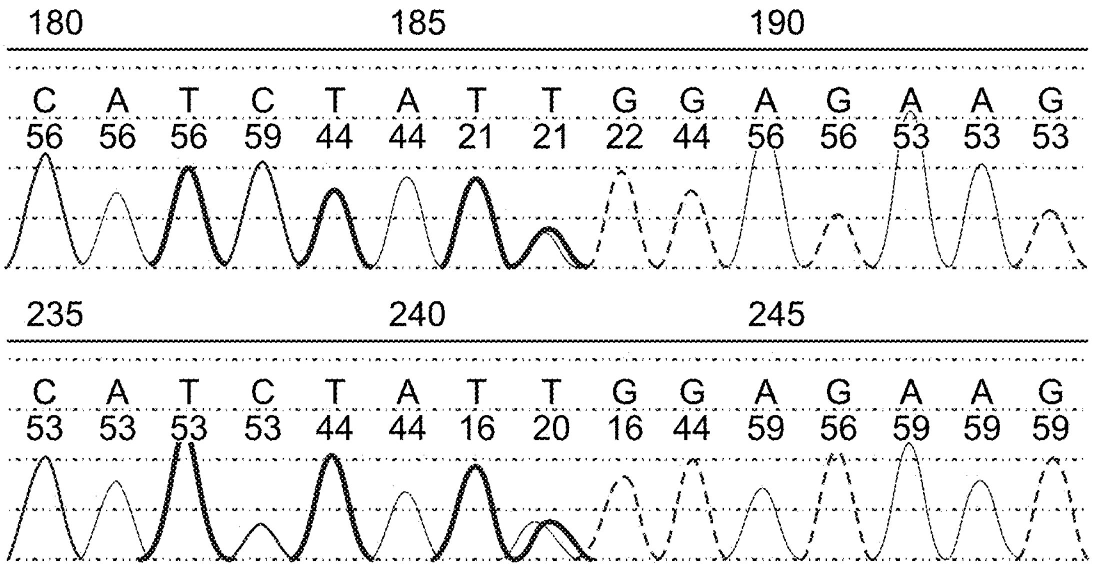


FIG. 17C

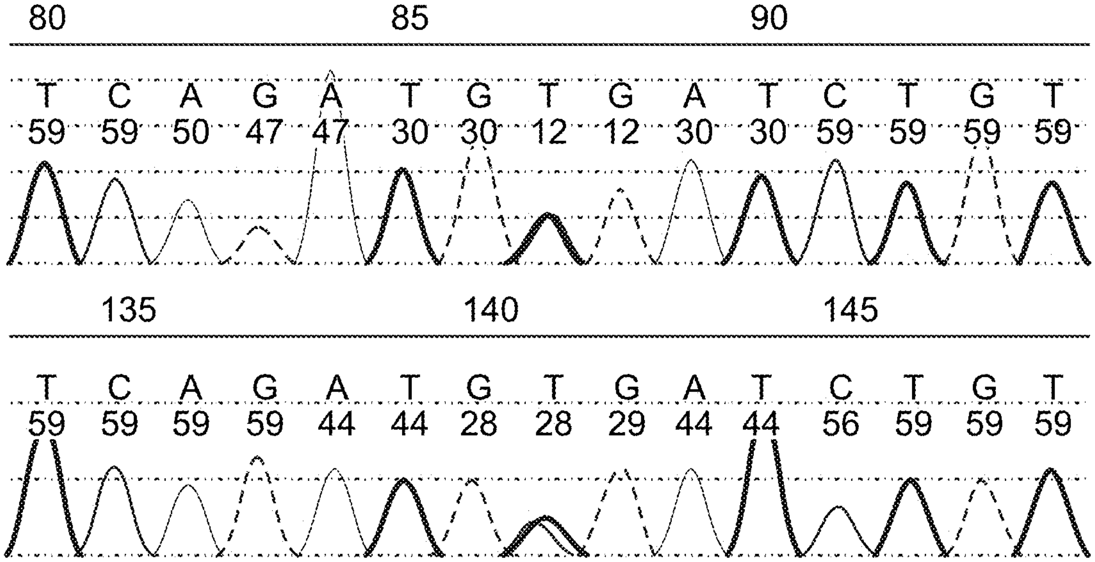


FIG. 17D

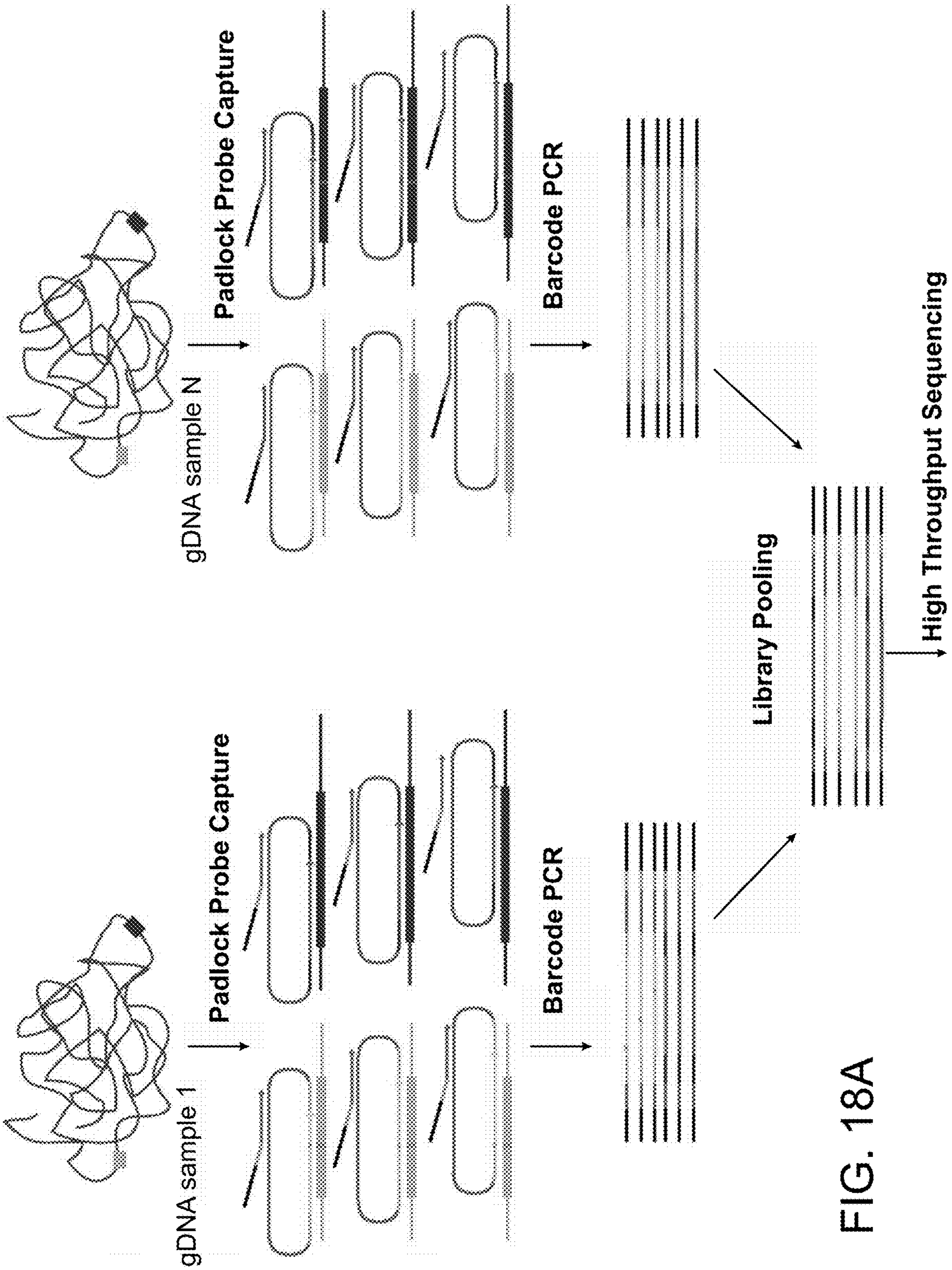
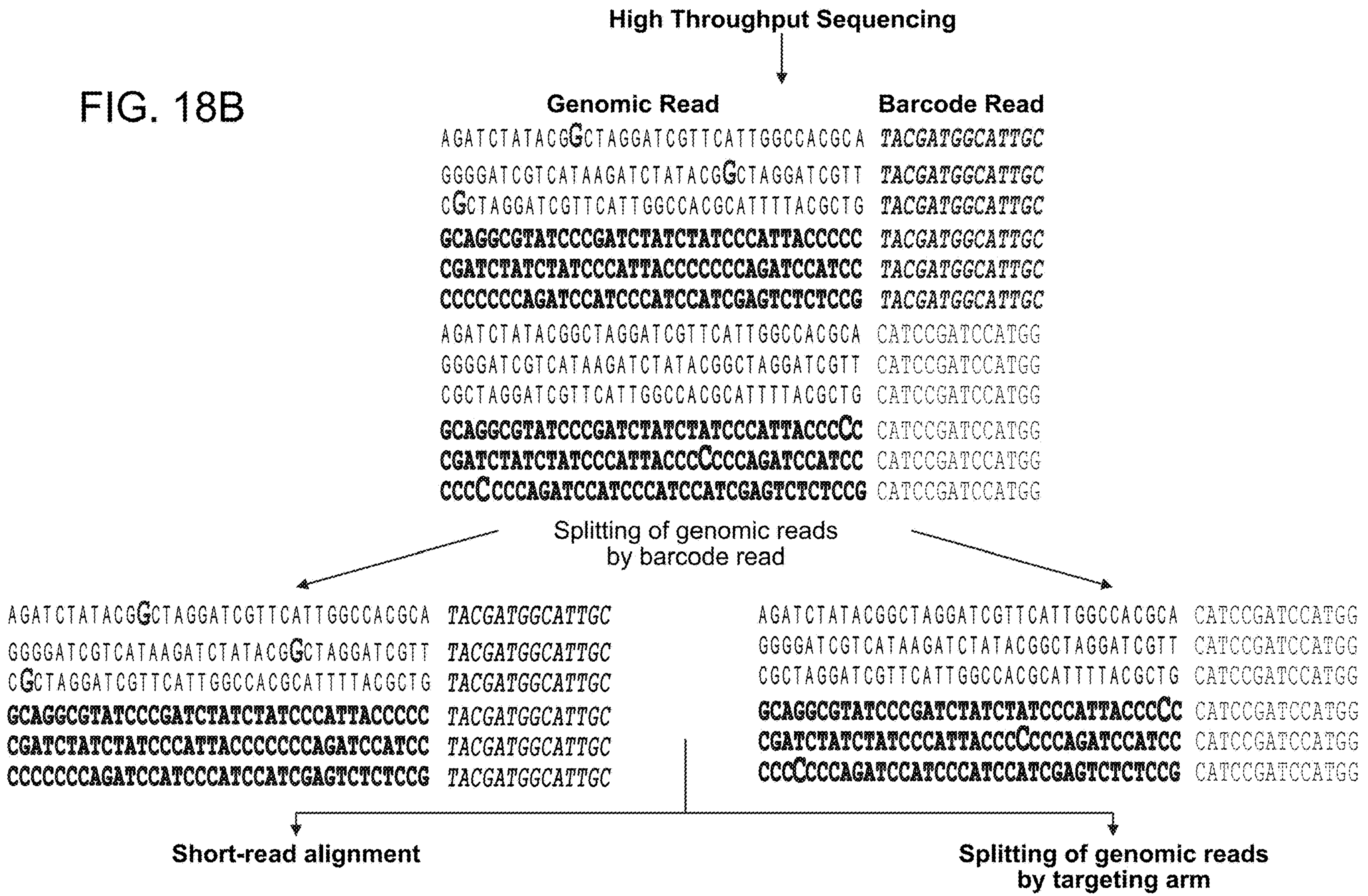

gDNA sample 1
Padlock Probe Capture
Barcode PCR
gDNA sample N
Padlock Probe Capture
Barcode PCR
Library Pooling
High Throughput Sequencing

FIG. 18A

FIG. 18B


High Throughput Sequencing
Genomic Read
Barcode Read
AGATCTATACGGCTAGGATCGTTCATTGGCCACGCA TACGATGGCATTGC
GGGGATCGTCATAAGATCTATACGGCTAGGATCGTT TACGATGGCATTGC
CGCTAGGATCGTTCATTGGCCACGCATTTTACGCTG TACGATGGCATTGC
GCAGGCGTATCCCGATCTATCTATCCCATTACCCCC TACGATGGCATTGC
CGATCTATCTATCCCATTACCCCCCCAGATCCATCC TACGATGGCATTGC
CCCCCCCAGATCCATCCCATCCATCGAGTCTCTCCG TACGATGGCATTGC
AGATCTATACGGCTAGGATCGTTCATTGGCCACGCA CATCCGATCCATGG
GGGGATCGTCATAAGATCTATACGGCTAGGATCGTT CATCCGATCCATGG
CGCTAGGATCGTTCATTGGCCACGCATTTTACGCTG CATCCGATCCATGG
GCAGGCGTATCCCGATCTATCTATCCCATTACCCCC CATCCGATCCATGG
CGATCTATCTATCCCATTACCCCCCCAGATCCATCC CATCCGATCCATGG
CCCCCCCAGATCCATCCCATCCATCGAGTCTCTCCG CATCCGATCCATGG
Splitting of genomic reads by barcode read
AGATCTATACGGCTAGGATCGTTCATTGGCCACGCA TACGATGGCATTGC
GGGGATCGTCATAAGATCTATACGGCTAGGATCGTT TACGATGGCATTGC
CGCTAGGATCGTTCATTGGCCACGCATTTTACGCTG TACGATGGCATTGC
GCAGGCGTATCCCGATCTATCTATCCCATTACCCCC TACGATGGCATTGC
CGATCTATCTATCCCATTACCCCCCCAGATCCATCC TACGATGGCATTGC
CCCCCCCAGATCCATCCCATCCATCGAGTCTCTCCG TACGATGGCATTGC
AGATCTATACGGCTAGGATCGTTCATTGGCCACGCA CATCCGATCCATGG
GGGGATCGTCATAAGATCTATACGGCTAGGATCGTT CATCCGATCCATGG
CGCTAGGATCGTTCATTGGCCACGCATTTTACGCTG CATCCGATCCATGG
GCAGGCGTATCCCGATCTATCTATCCCATTACCCCC CATCCGATCCATGG
CGATCTATCTATCCCATTACCCCCCCAGATCCATCC CATCCGATCCATGG
CCCCCCCAGATCCATCCCATCCATCGAGTCTCTCCG CATCCGATCCATGG
Short-read alignment
Splitting of genomic reads by targeting arm

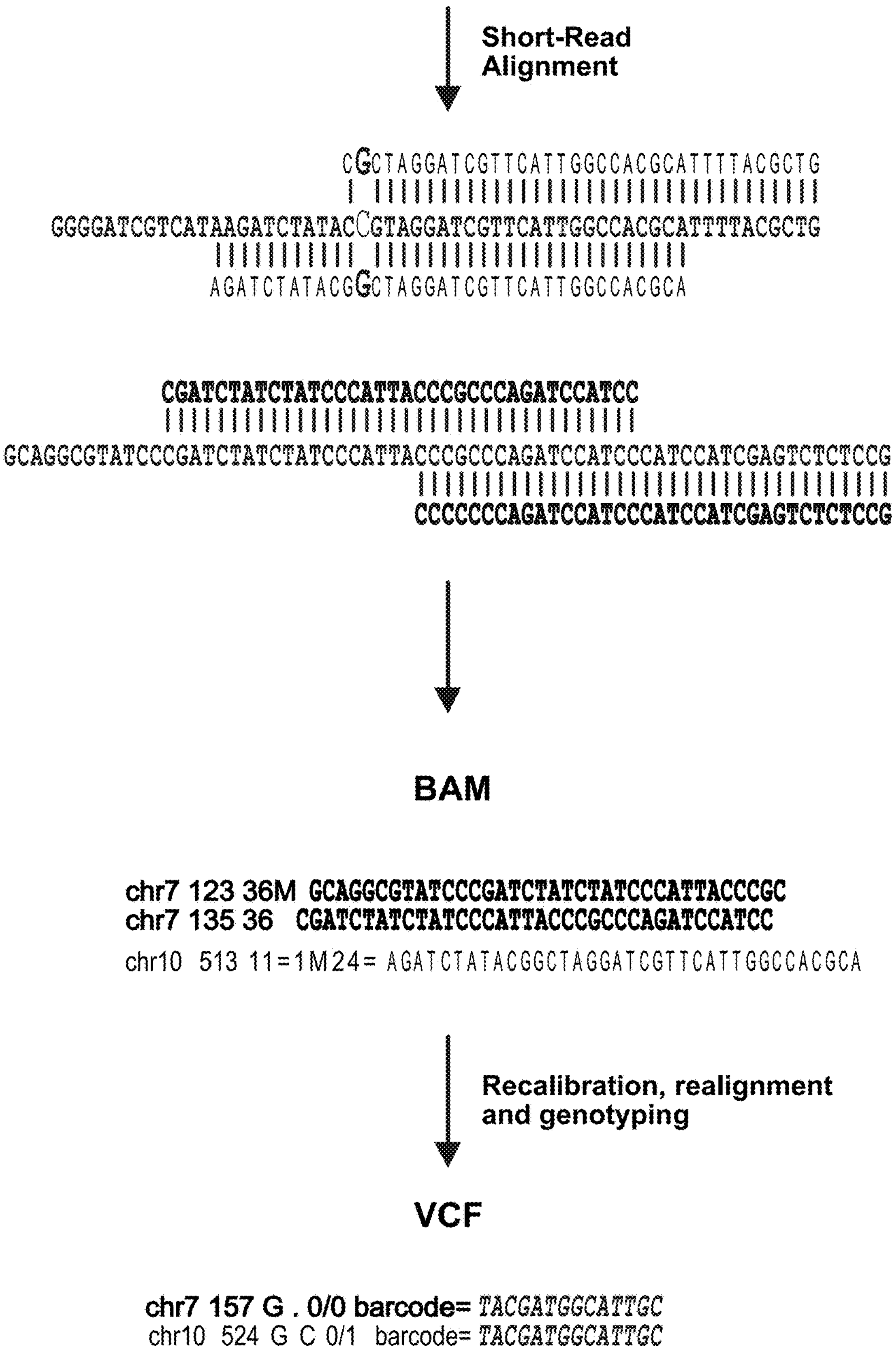

Short-Read Alignment
cGCTAGGATCGTTCATTGGCCACGCATTTTACGCTG
GGGGATCGTCATAAGATCTATACCGTAGGATCGTTCATTGGCCACGCATTTTACGCTG
AGATCTATACGGCTAGGATCGTTCATTGGCCACGCA
CGATCTATCTATCCCATTACCCGCCCAGATCCATCC
GCAGGCGTATCCCGATCTATCTATCCCATTACCCGCCCAGATCCATCCCATCCATCGAGTCTCTCCG
CCCCCCCAGATCCATCCCATCCATCGAGTCTCTCCG
BAM
chr7 123 36M GCAGGCGTATCCCGATCTATCTATCCCATTACCCGC
chr7 135 36 CGATCTATCTATCCCATTACCCGCCCAGATCCATCC
chr10 513 11=1M24= AGATCTATACGGCTAGGATCGTTCATTGGCCACGCA
Recalibration, realignment and genotyping
VCF
chr7 157 G . 0/0 barcode= TACGATGGCATTGC
chr10 524 G C 0/1 barcode= TACGATGGCATTGC

FIG. 18C

FIG. 18D

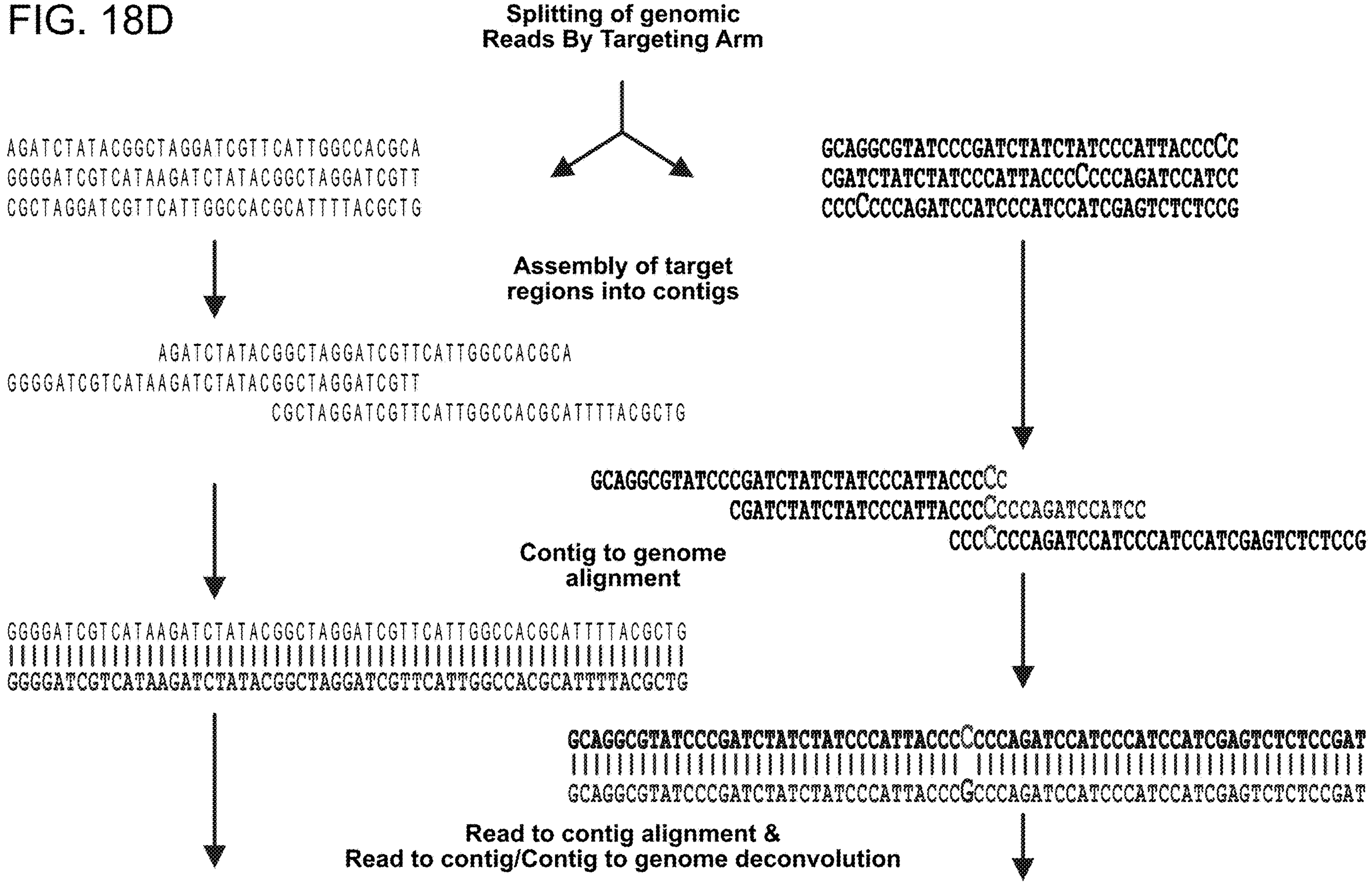
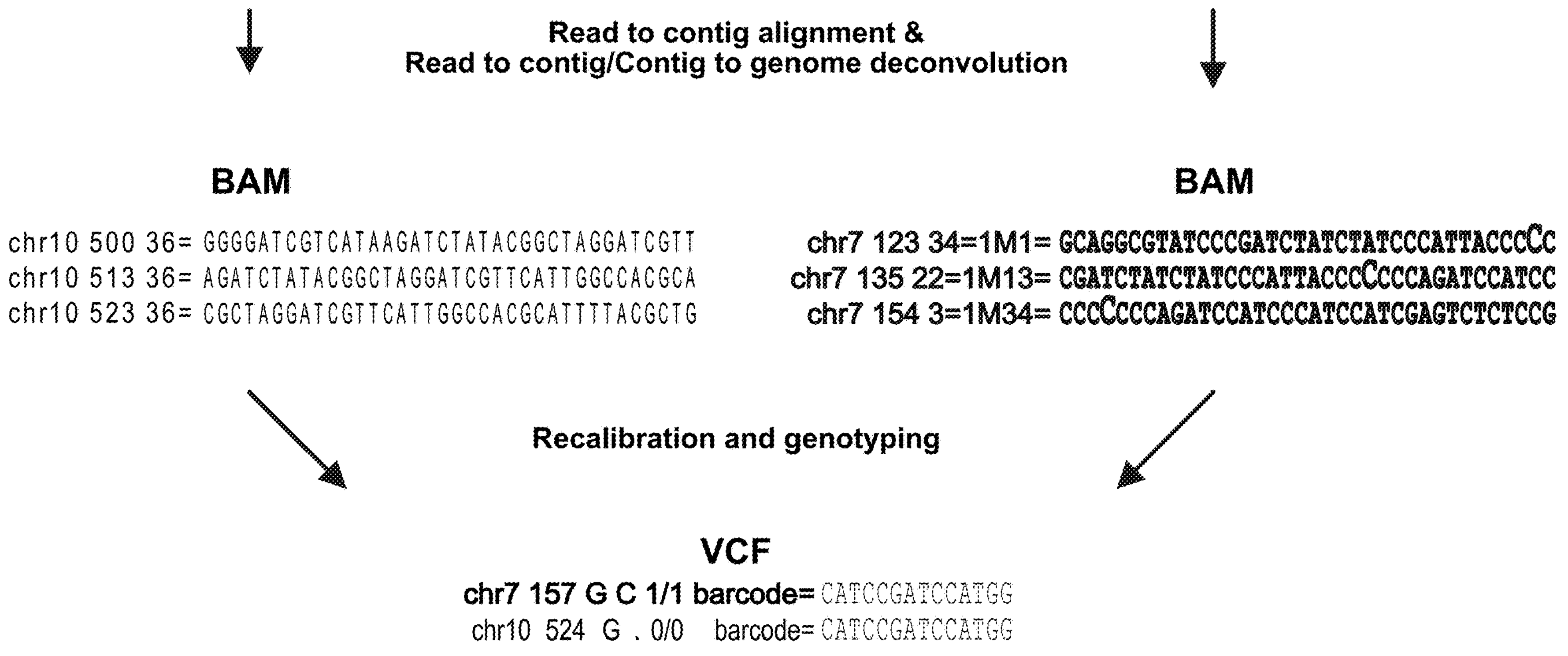

Splitting of genomic Reads By Targeting Arm
AGATCTATACGGCTAGGATCGTTCATTGGCCACGCA
GGGGATCGTCATAAGATCTATACGGCTAGGATCGTT
CGCTAGGATCGTTCATTGGCCACGCATTTTACGCTG
GCAGGCGTATCCCGATCTATCTATCCCATTACCCCc
CGATCTATCTATCCCATTACCCCCCCAGATCCATCC
CCCCCCCAGATCCATCCCATCCATCGAGTCTCTCCG
Assembly of target regions into contigs
AGATCTATACGGCTAGGATCGTTCATTGGCCACGCA
GGGGATCGTCATAAGATCTATACGGCTAGGATCGTT
CGCTAGGATCGTTCATTGGCCACGCATTTTACGCTG
GCAGGCGTATCCCGATCTATCTATCCCATTACCCCc
CGATCTATCTATCCCATTACCCCCCCAGATCCATCC
CCCCCCCAGATCCATCCCATCCATCGAGTCTCTCCG
Contig to genome alignment
GGGGATCGTCATAAGATCTATACGGCTAGGATCGTTCATTGGCCACGCATTTTACGCTG
GGGGATCGTCATAAGATCTATACGGCTAGGATCGTTCATTGGCCACGCATTTTACGCTG
GCAGGCGTATCCCGATCTATCTATCCCATTACCCCCCCAGATCCATCCCATCCATCGAGTCTCTCCGAT
GCAGGCGTATCCCGATCTATCTATCCCATTACCCGCCCAGATCCATCCCATCCATCGAGTCTCTCCGAT
Read to contig alignment &
Read to contig/Contig to genome deconvolution

FIG. 18E



Read to contig alignment &
Read to contig/Contig to genome deconvolution

BAM

chr10 500 36= GGGGATCGTCATAAGATCTATACGGCTAGGATCGTT
chr10 513 36= AGATCTATACGGCTAGGATCGTTCATTGGCCACGCA
chr10 523 36= CGCTAGGATCGTTCATTGGCCACGCATTTTACGCTG

BAM

chr7 123 34=1M1= GCAGGCGTATCCCGATCTATCTATCCCATTACCCCC
chr7 135 22=1M13= CGATCTATCTATCCCATTACCCCCCCAGATCCATCC
chr7 154 3=1M34= CCCCCCCAGATCCATCCCATCCATCGAGTCTCTCCG

Recalibration and genotyping

VCF

chr7 157 G C 1/1 barcode= CATCCGATCCATGG
chr10 524 G . 0/0 barcode= CATCCGATCCATGG

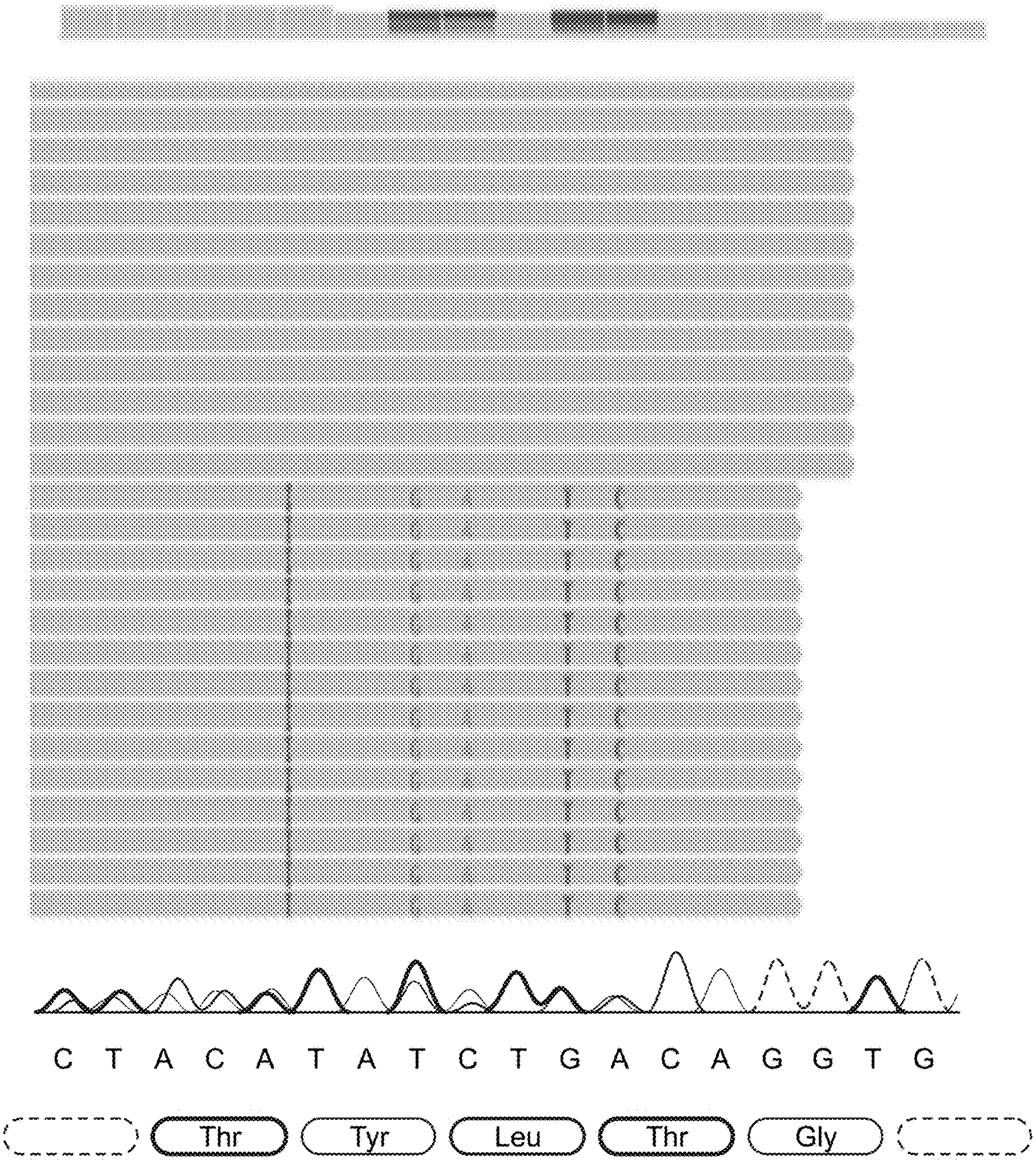
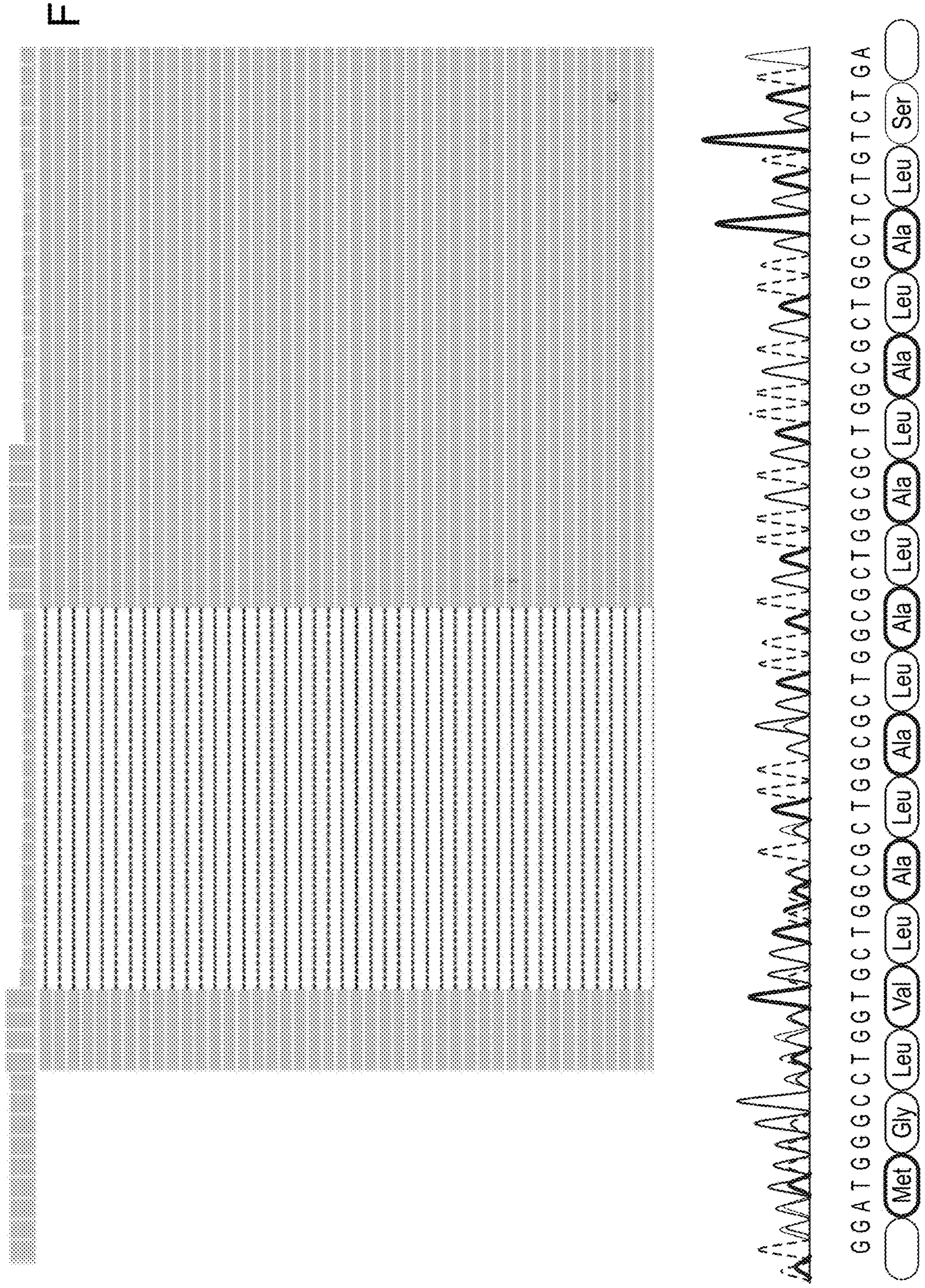


FIG. 19A

FIG. 19B


GGATGGGCCCTGGTGCTGGCGCTGGCGCTGGCGCTGGCGCTGGCGCTGGCGCTGGCTCTGTCTGA
Met
Gly
Leu
Val
Leu
Ala
Leu
Ala
Leu
Ala
Leu
Ala
Leu
Ala
Leu
Ala
Leu
Ser

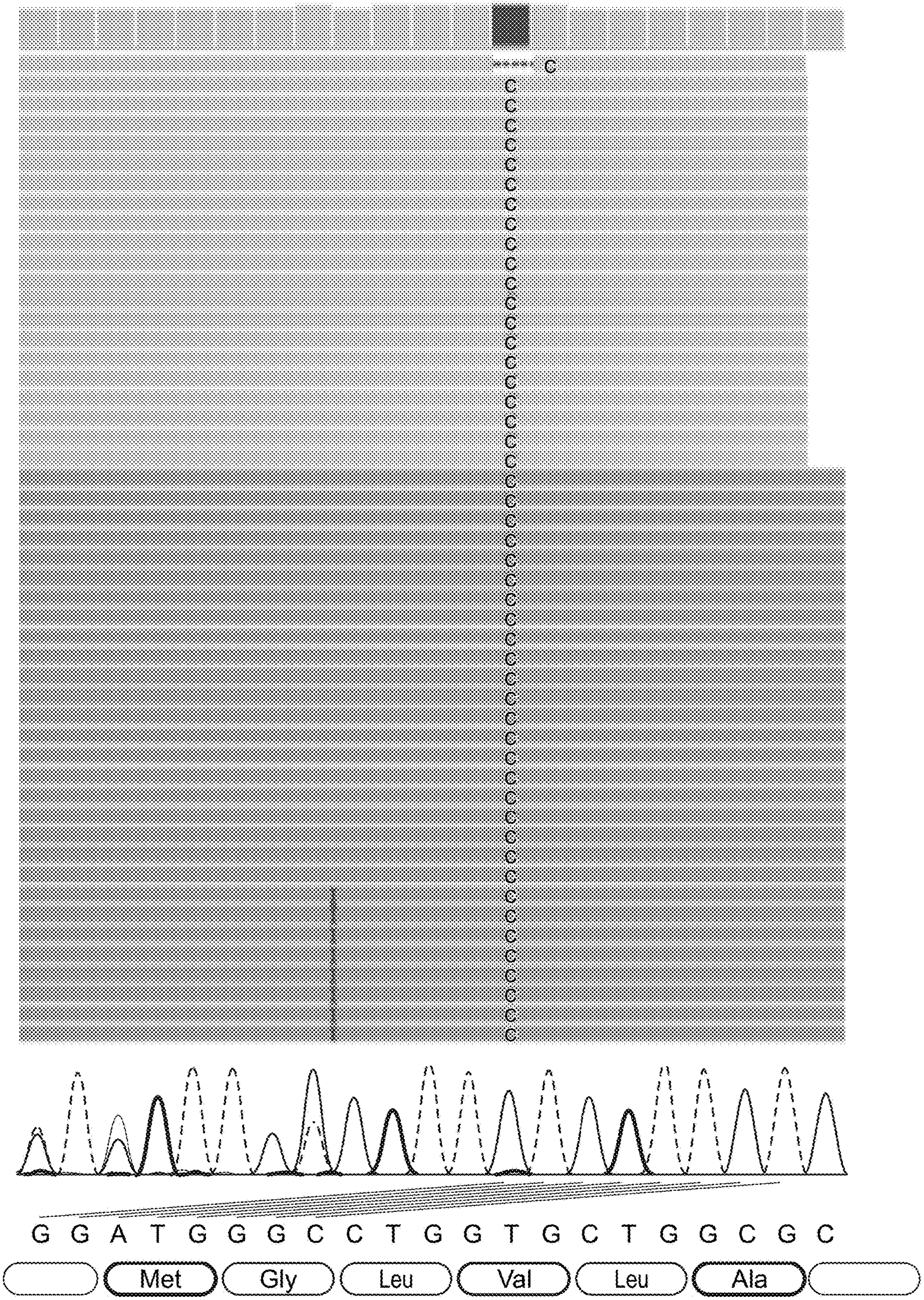


FIG. 19C

FIG. 19D

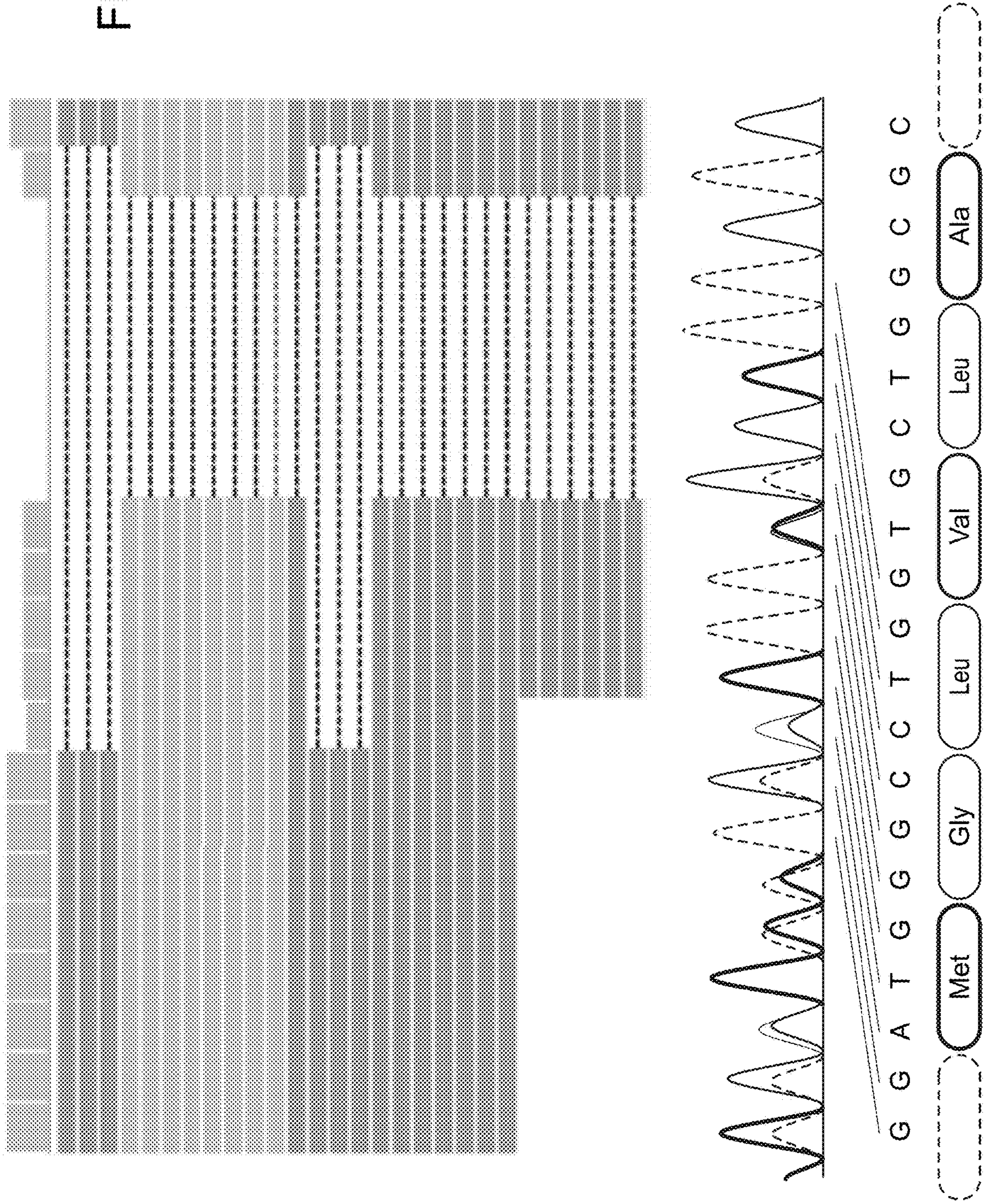


FIG. 20A-1

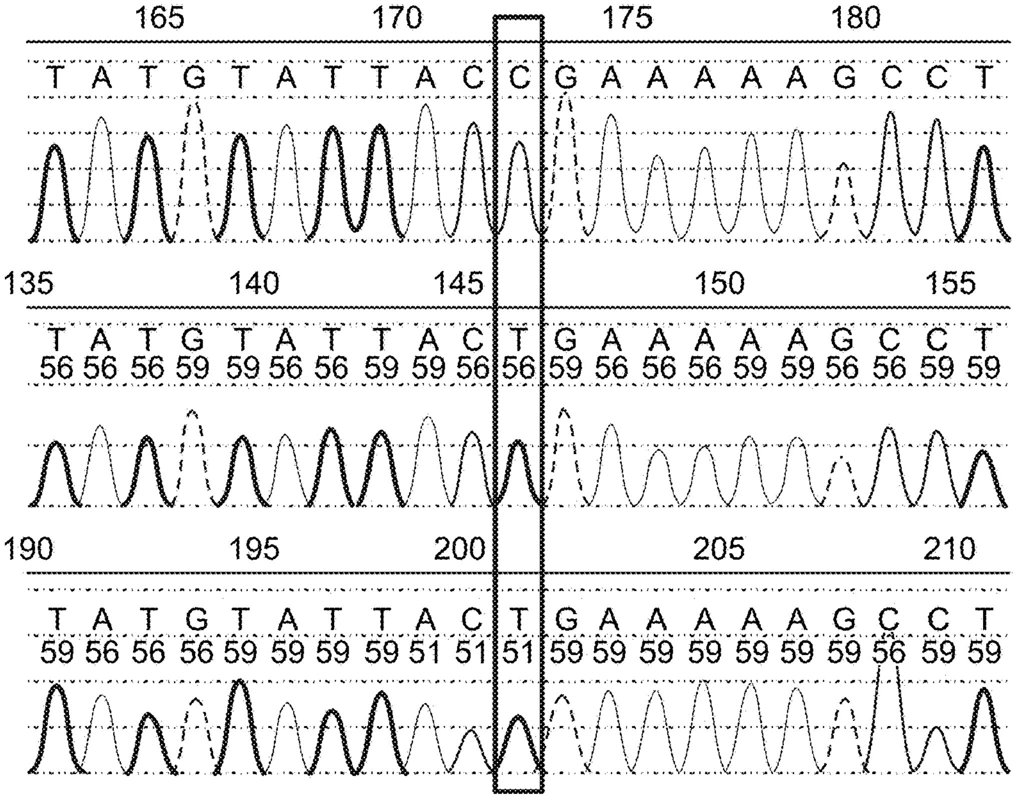
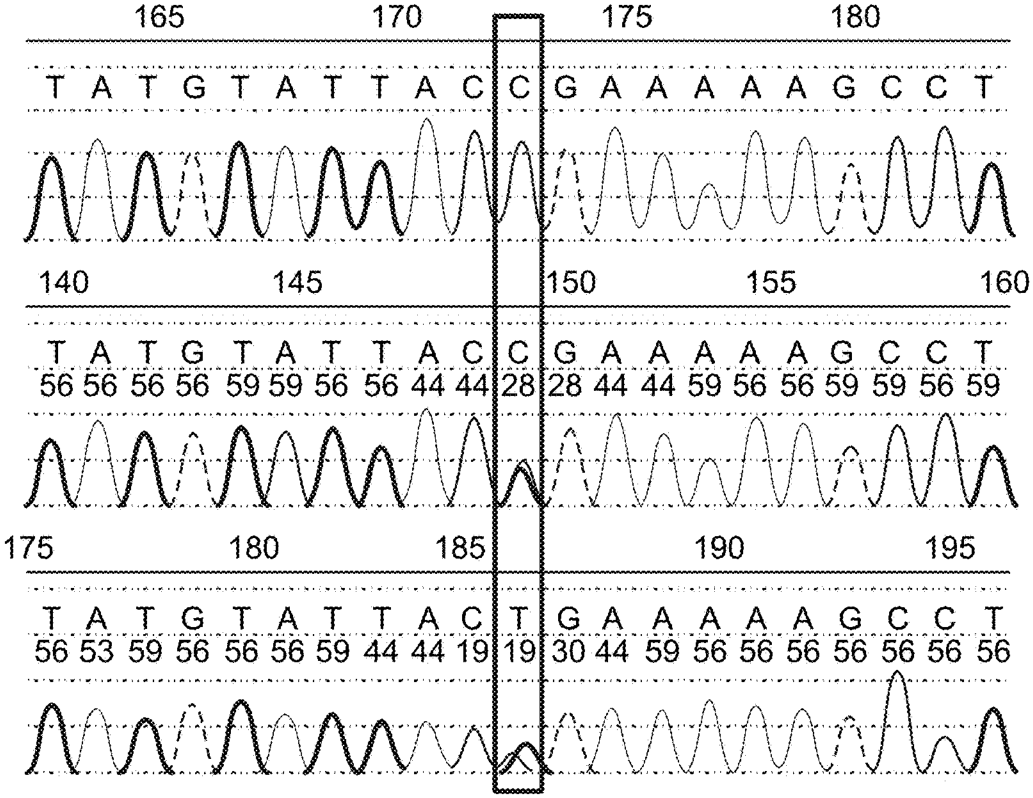


FIG. 20A-2



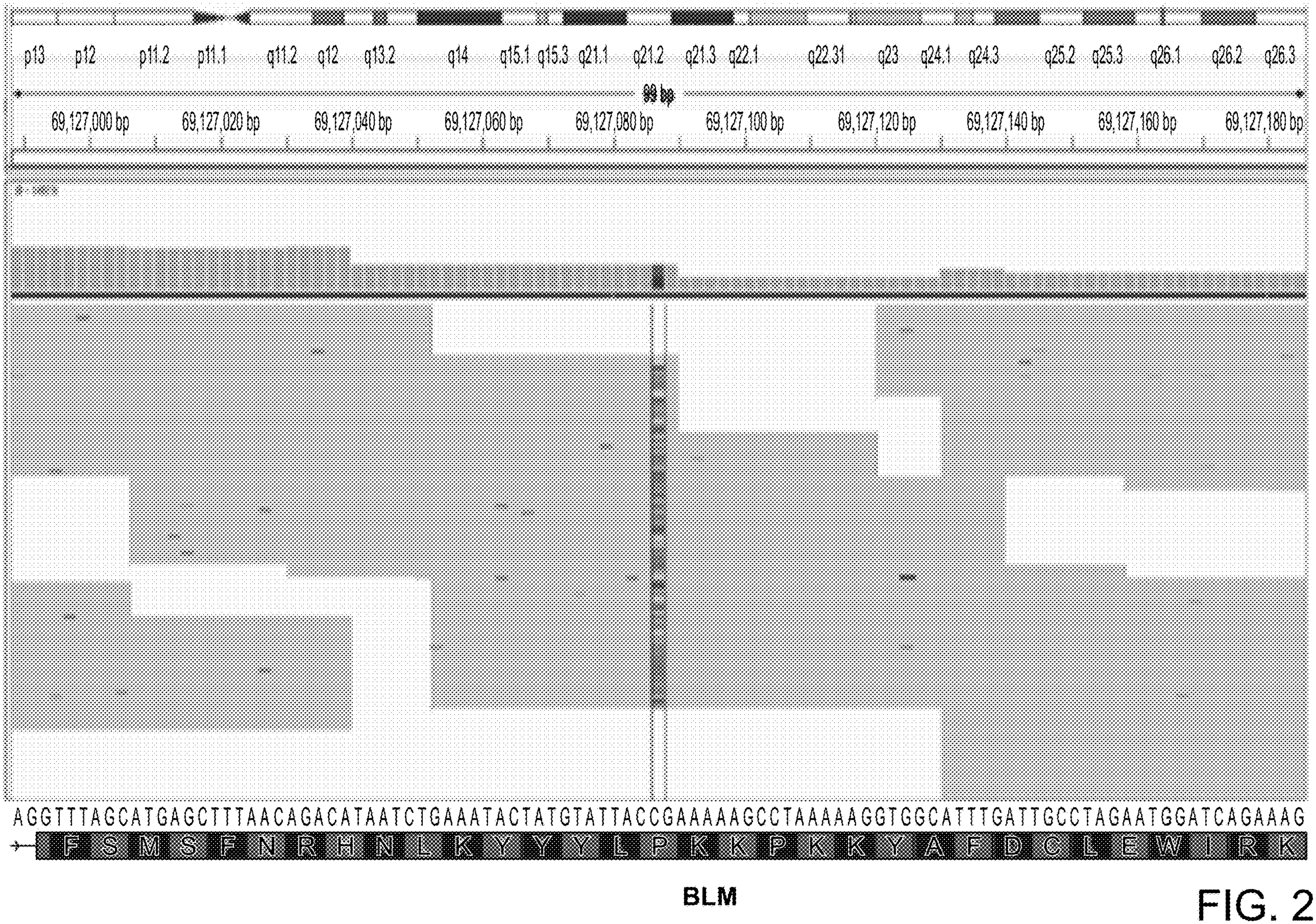

p13
p12
p11.2
p11.1
q11.2
q12
q13.2
q14
q15.1
q15.3
q21.1
q21.2
q21.3
q22.1
q22.31
q23
q24.1
q24.3
q25.2
q25.3
q26.1
q26.2
q26.3
99 bp
69,127,000 bp
69,127,020 bp
69,127,040 bp
69,127,060 bp
69,127,080 bp
69,127,100 bp
69,127,120 bp
69,127,140 bp
69,127,160 bp
69,127,180 bp
AGGTTTAGCATGAGCTTTAACAGACATAATCTGAAATACTATGTATTACCGAAAAAGCCTAAAAAGGTGGCATTTGATTGCCTAGAATGGATCAGAAAG
F S M S F N R H N L K Y Y Y L P K K P K K Y A F D C L E W I R K
BLM

FIG. 20A-3

FIG. 20B-1

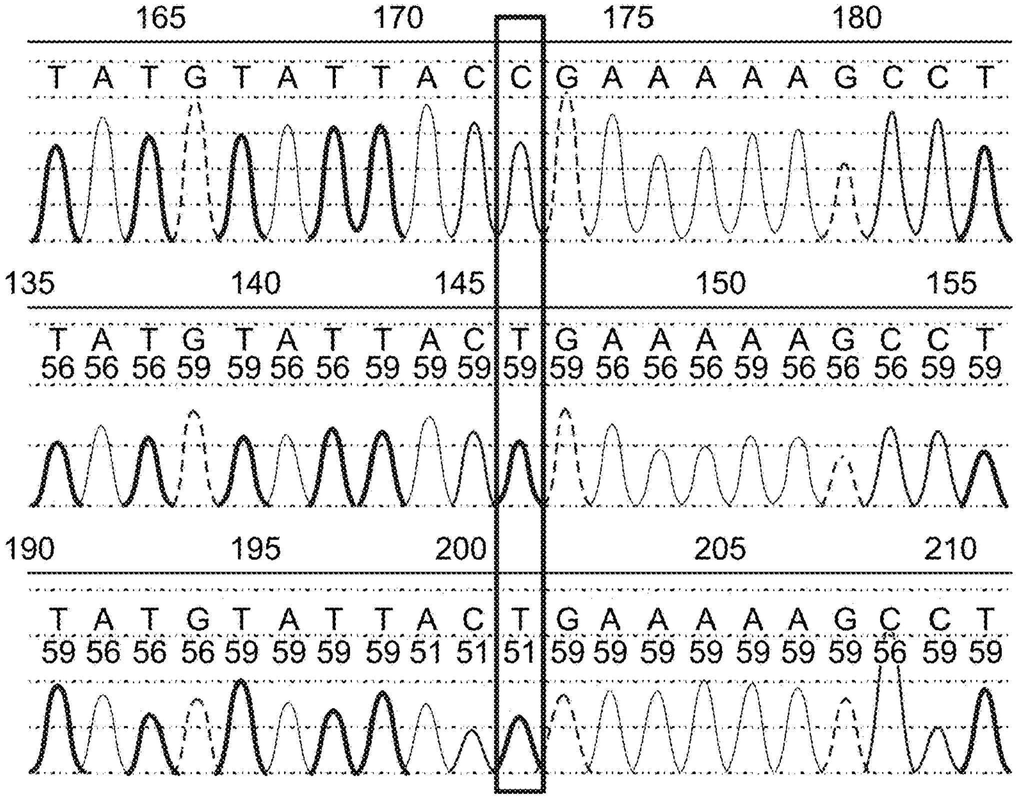
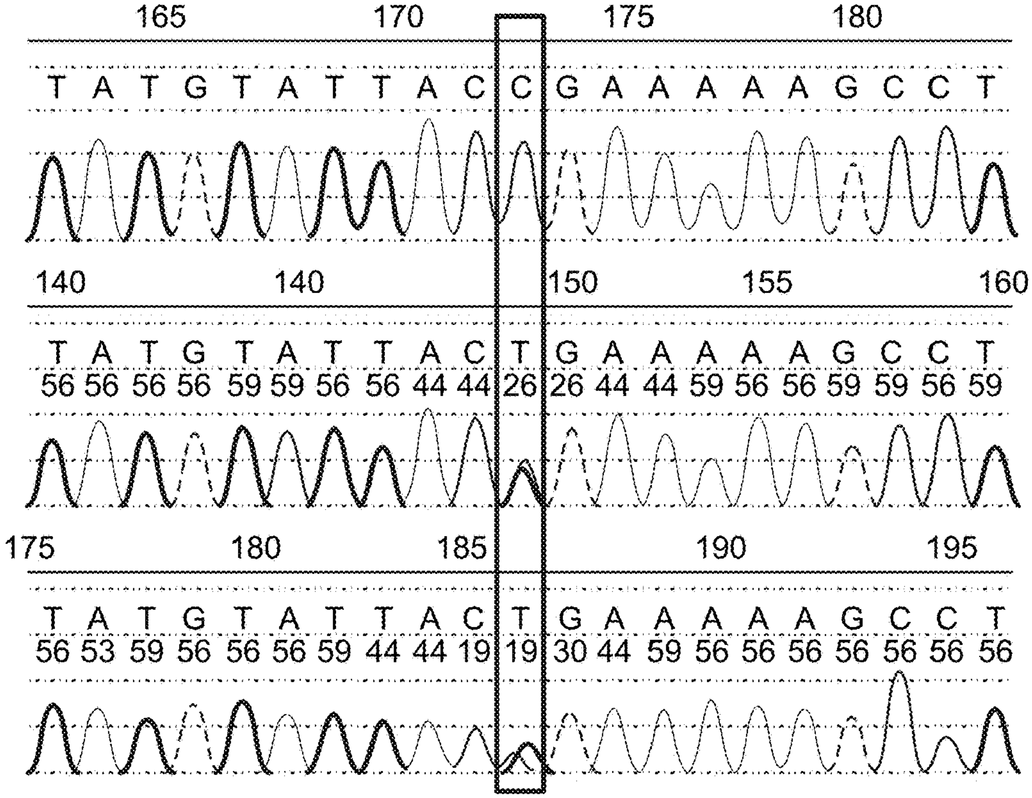


FIG. 20B-2



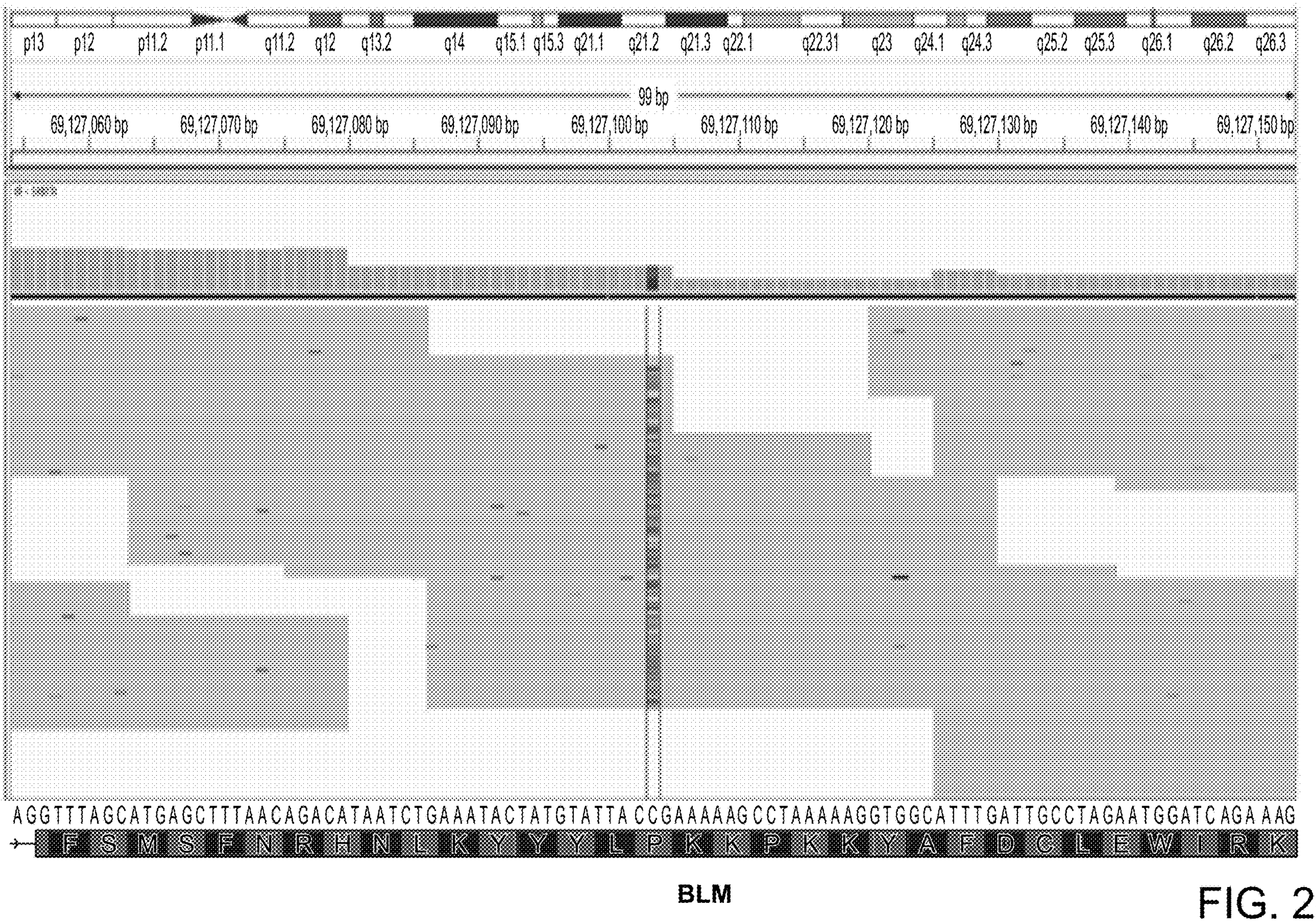

p13
p12
p11.2
p11.1
q11.2
q12
q13.2
q14
q15.1
q15.3
q21.1
q21.2
q21.3
q22.1
q22.31
q23
q24.1
q24.3
q25.2
q25.3
q26.1
q26.2
q26.3
99 bp
69,127,060 bp
69,127,070 bp
69,127,080 bp
69,127,090 bp
69,127,100 bp
69,127,110 bp
69,127,120 bp
69,127,130 bp
69,127,140 bp
69,127,150 bp
AGGTTTAGCATGAGCTTTAACAGACATAATCTGAAATACTATGTATTA CCGAAAAAGCCTAAAAAGGTGGCATTTGATTGCCTAGAATGGATCAGA AAG
F S M S F N R H N L K Y Y Y L P K K P K K Y A F D C L E W I R K
BLM

FIG. 20B-3

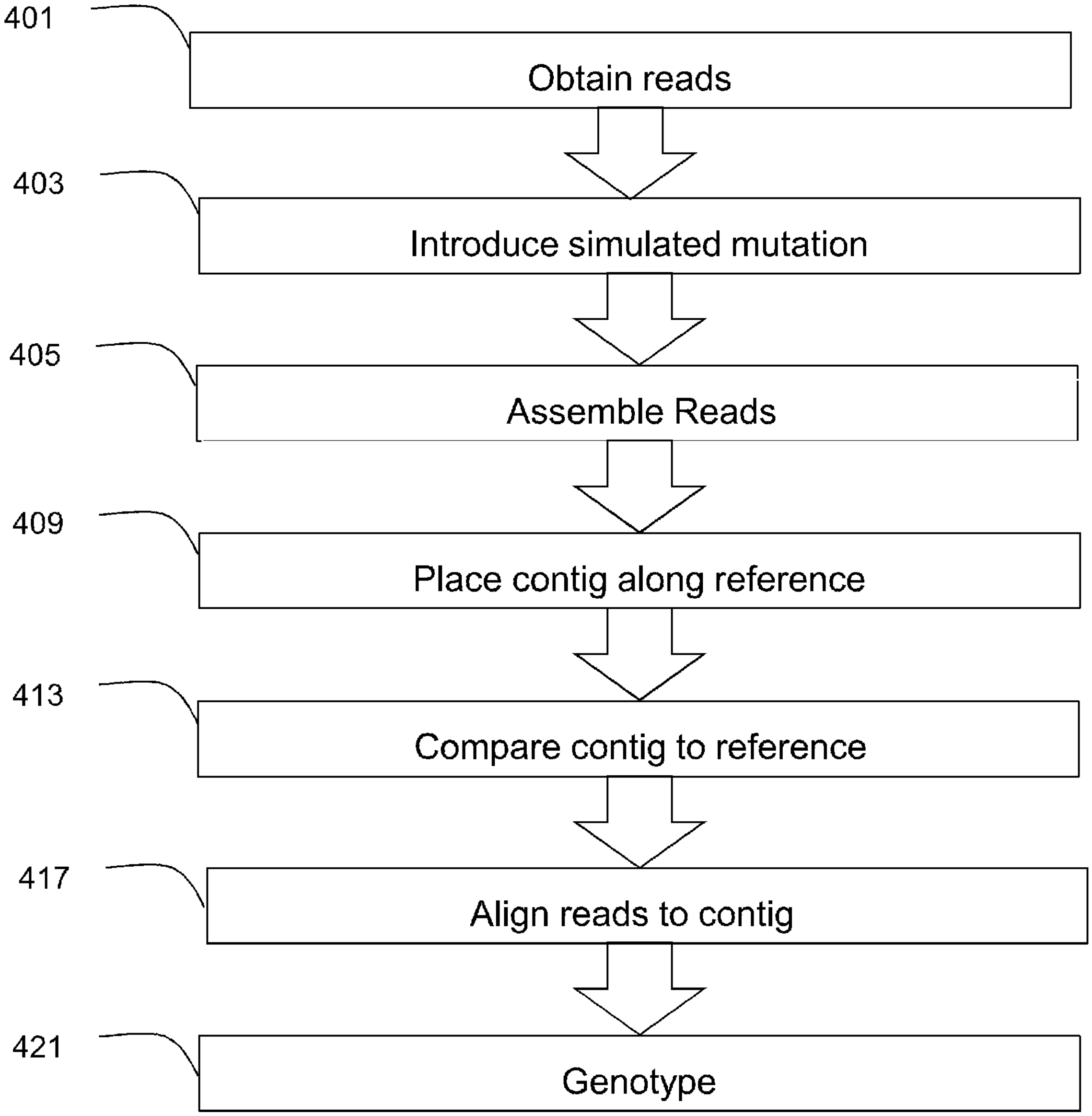

401
Obtain reads
403
Introduce simulated mutation
405
Assemble Reads
409
Place contig along reference
413
Compare contig to reference
417
Align reads to contig
421
Genotype

FIG. 21

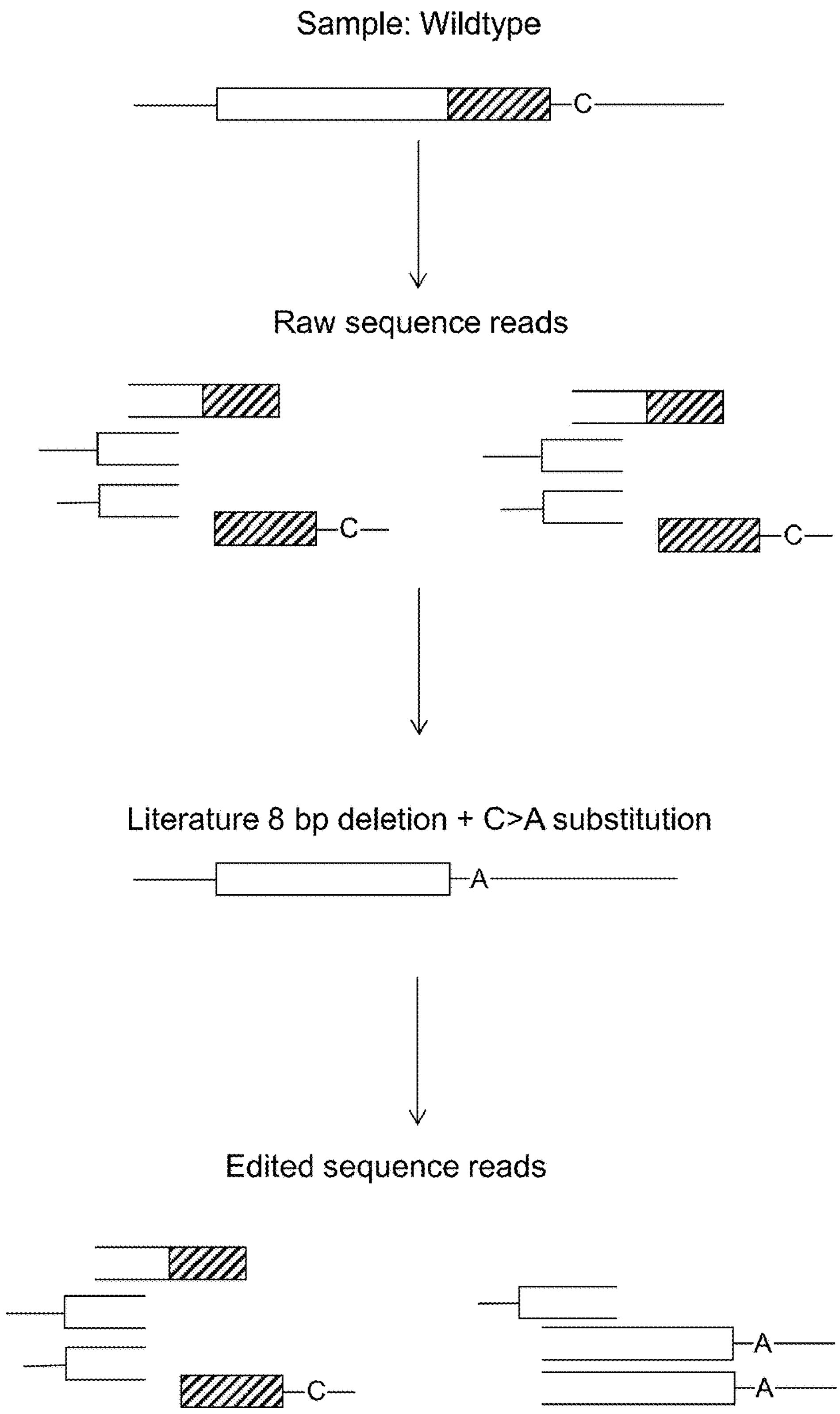

Sample: Wildtype
C
Raw sequence reads
C
C
Literature 8 bp deletion + C>A substitution
A
Edited sequence reads
C
A
A

FIG. 22

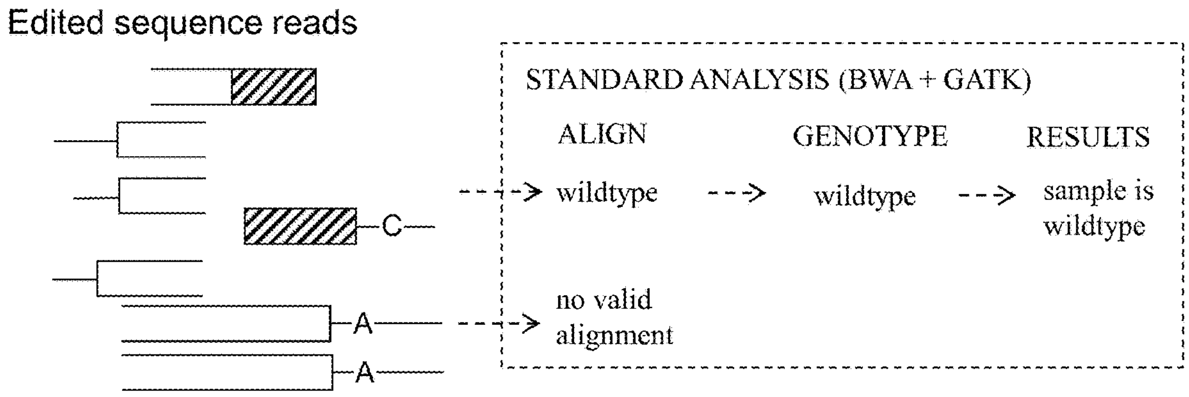
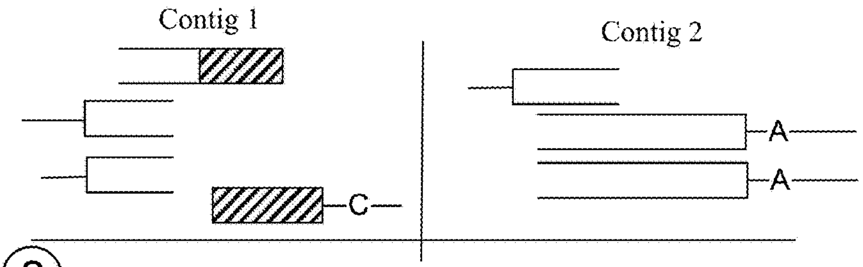

Edited sequence reads
C
A
A
STANDARD ANALYSIS (BWA + GATK)
ALIGN
GENOTYPE
RESULTS
wildtype
wildtype
sample is wildtype
no valid alignment

FIG. 23

1 Assemble into contigs: 2 are found (180 bp)



2 Align contigs: both align to same reference position, but one contains a deletion and substitution, the other does not.

	Position	Cigar
Allele 1:	Chr 7: 11608523	180M
Allele 2:	Chr 7: 11608523	75M8D105M

3 Align raw reads to contigs (raw reads are 80 bp)

read 1	Contig1:1	80M
read 2	Contig2:1	80M
read 3	Contig2:50	80M
read 4	Contig2:100	80M
read 5	Contig1:50	80M
...		
read 6	Contig1:100	80M

4 Map raw read alignments from contig space to original reference space (position & cigar)

	CONTIG		REFERENCE	
	Position	Cigar	Position	Cigar
read 1	1:1	80M	Chr7:11608523	85M
read 2	2:1	80M	Chr7:11608523	75M8D5M
read 3	2:50	80M	Chr7:11608573	25M8D55M
read 4	2:100	80M	Chr7:11608631	80M
read 5	1:50	80M	Chr7:11608573	80M
…				
read N	1:100	80M	Chr7:11608623	80M

5 Perform genotyping using translated, aligned reads from #4, including raw quality scores for substitutions

FIG. 24

METHODS AND COMPOSITIONS FOR EVALUATING GENETIC MARKERS

RELATED APPLICATIONS

The application claims the benefit of and priority to U.S. provisional application Ser. No. 61/789,164, filed Mar. 15, 2013, and is a continuation-in-part of U.S. application Ser. No. 13/266,862, which has a Section 371 (c) date of Mar. 13, 2012 and is a National Stage Entry of PCT application Ser. No. PCT/US10/01293, filed Apr. 30, 2010, which claims priority to and the benefit of U.S. provisional application Ser. No. 61/174,470, filed Apr. 30, 2009, U.S. provisional application Ser. No. 61/178,923, filed May 15, 2009, U.S. provisional application Ser. No. 61/179,358, filed May 18, 2009, and U.S. provisional application Ser. No. 61/182,089, filed May 28, 2009. The entire contents of each of these applications are incorporated to herein by reference.

SEQUENCE LISTING

The instant application contains a Sequence Listing which has been submitted in ASCII format via EFS-Web and is hereby incorporated by reference in its entirety. Said ASCII copy, created on Aug. 23, 2013, is named GSGE_002_03US_Sequence_Listing.txt and is 1,922,061 bytes in size.

FIELD OF INVENTION

The invention relates to methods and compositions for determining genotypes in patient samples.

BACKGROUND OF THE INVENTION

Information about the genotype of a subject is becoming more important and relevant for a range of healthcare decisions as the genetic basis for many diseases, disorders, and physiological characteristics is further elucidated. Medical advice is increasingly personalized, with individual decisions and recommendations being based on specific genetic information. Information about the type and number of alleles at one or more genetic loci impacts disease risk, prognosis, therapeutic options, and genetic counseling amongst other healthcare considerations.

For cost-effective and reliable medical and reproductive counseling on a large scale, it is important to be able correctly and unambiguously identify the allelic status for many different genetic loci in many subjects.

Numerous technologies have been developed for detecting and analyzing nucleic acid sequences from biological samples. These technologies can be used to genotype subjects and determine the allelic status of any locus of interest. However, they are not sufficiently robust and cost-effective to be scaled up for reliable high throughput analysis of many genetic loci in large numbers of patients. The frequency of incorrect or ambiguous calls is too high for current technology to manage large numbers of patient samples without involving expensive and time-consuming steps to resolve uncertainties and provide confidence in the information output.

SUMMARY OF THE INVENTION

Aspects of the invention relate to preparative and analytical methods and compositions for evaluating genotypes,

and in particular, for determining the allelic identity (or identities in a diploid organism) of one or more genetic loci in a subject.

Aspects of the invention are based, in part, on the identification of different sources of ambiguity and error in genetic analyses, and, in part, on the identification of one or more approaches to avoid, reduce, recognize, and/or resolve these errors and ambiguities at different stages in a genetic analysis.

According to aspects of the invention, certain types of genetic information can be under-represented or over-represented in a genetic analysis due to a combination of stochastic variation and systematic bias in any of the preparative stages (e.g., capture, amplification, etc.), determining stages (e.g., allele-specific detection, sequencing, etc.), data interpretation stages (e.g., determining whether the assay information is sufficient to identify a subject as homozygous or heterozygous), and/or other stages.

According to aspects of the invention, error or ambiguity may be apparent in a genetic analysis, but not readily resolved without running additional samples or more expensive assays (e.g., array-based assays may report no-calls due to noisy/low signal). According to further aspects of the invention, error or ambiguity may not be accounted for in a genetic analysis and incorrect base calls may be made even when the evidence for them is limited and/or not statistically significant (e.g., next-generation sequencing technologies may report base calls even if the evidence for them is not statistically significant). According to further aspects of the invention error or ambiguity may be problematic for a multi-step genetic analysis because it is apparent but not readily resolved in one or more steps of the analysis and not apparent or accounted for in other steps of the analysis.

In some embodiments, sources of error and ambiguity in one or more steps can be addressed by capturing and/or interrogating each target locus of interest with one or more sets of overlapping probes that are designed to overcome any systematic bias or stochastic effects that may impact the complexity and/or fidelity of the genetic information that is generated.

In some embodiments, sources of error and ambiguity in one or more steps can be addressed by capturing and/or interrogating each target locus of interest with at least one set of probes, wherein different probes are labeled with different identifiers that can be used to track the assay reactions and determine whether certain types of genetic information are under-represented or over-represented in the information that is generated.

In some embodiments, errors and ambiguities associated with the analysis of regions containing large numbers of sequence repeats are addressed by systematically analyzing frequencies of certain nucleic acids at particular stages in an assay (e.g., at a to capture, sequencing, or detection stage). It should be appreciated that such techniques may be particularly useful in the context of a standardized protocol that is designed to allow many different loci to be evaluated in parallel without requiring different assay procedures for each locus. In some embodiments, the use of a single detection modality (e.g., sequencing) to assay multiple types of genetic lesions (e.g., point mutations, insertions/deletions, length polymorphisms) is advantageous in the clinical setting. In some embodiments of the invention, methods are provided that facilitate the use of multiple sample preparation steps in parallel, coupled with multiple analytical processes following sequence detection. Thus, in some embodiments of the invention, an improved workflow is provided that reduces error and uncertainty when simultaneously assaying different types of genetic lesions across multiple loci in multiple patients.

In some embodiments, aspects of the invention provide methods for overcoming preparative and/or analytical bias by combining two or more techniques, each having a different bias (e.g., a known bias towards under-representation or over-representation of one or more types of sequences), and using the resulting data to determine a genetic call for a subject with greater confidence.

It should be appreciated that in some embodiments, aspects of the invention relate to multiplex diagnostic methods. In some embodiments, multiplex diagnostic methods comprise capturing a plurality of genetic loci in parallel (e.g., one or more genetic loci from Table 1). In some embodiments, the genetic loci possess one or more polymorphisms (e.g., one or more polymorphisms from Table 2) the genotypes of which correspond to disease causing alleles.

Accordingly, in some embodiments, the disclosure provides methods for assessing multiple heritable disorders in parallel. In some embodiments, methods are provided for diagnosing multiple heritable disorders in parallel at a pre-implantation, prenatal, perinatal, or postnatal stage. In some embodiments, the disclosure provides methods for analyzing multiple genetic loci (e.g., a plurality of target nucleic acids selected from Table 1) from a patient sample, such as a blood, pre-implantation embryo, chorionic villus or amniotic fluid sample, or other sample (e.g., other biological fluid or tissue sample such as a biopsy sample) as aspects of the invention are not limited in this respect.

Other samples may include tumor tissue or circulating tumor cells. In some embodiments, a patient sample (e.g., a tumor tissue or cell sample) is mosaic for one or more mutations of interest, and thus, may require higher sensitivity than is needed for a germline mutation analysis. In some embodiments, a sample comprises cells from a non-host organism (e.g., bacterial or viral infections in a human subject) or a sample for environmental monitoring (e.g., bacterial, viral, fungal composition of a soil, water, or air sample).

Accordingly, in some embodiments, aspects of the methods disclosed herein relate to genotyping a polymorphism of a target nucleic acid. In some embodiments, the genotyping may comprise determining that one or more alleles of the target nucleic acid are heterozygous or homozygous. In further embodiments, the genotyping may comprise determining the sequence of a polymorphism and comparing that sequence to a control sequence that is indicative of a disease risk. In some embodiments, the polymorphism is selected from a locus in Table 1 or Table 2. However, it should be appreciated that any locus associated with a disease or condition of interest may be used.

In some embodiments, a diagnosis, prognosis, or disease risk assessment is provided to a subject based on a genotype determined for that subject at one or more genetic loci (e.g., based on the analysis of a biological sample obtained from that subject). In some embodiments, an assessment is provided to a couple, based on their respective genotypes at one or more genetic loci, of the risk of their having one or more children having a genotype associated with a disease or condition (e.g., a homozygous or heterozygous genotype associated with a disease or condition). In some embodiments, a subject or a couple may seek genetic or reproductive counseling in connection with a genotype determined according to embodiments of the invention. In some embodiments, genetic information from a tumor or circulating tumor cells is used to determine prognosis and guide selection of appropriate drugs/treatments.

It should be appreciated that any of the methods or compositions described herein may be used in combination with any of the medical evaluations associated with one or more genetic loci as described herein.

In some embodiments, aspects of the invention provide effective methods for overcoming challenges associated with systematic errors (bias) and/or stochastic effects in multiplex genomic capture and/or analysis (including sequencing analysis). In some embodiments, aspects of the invention are useful to avoid, reduce and/or account for variability in one or more sampling and/or analytical steps. For example, in some embodiments, variability in target nucleic acid representation and unequal sampling of heterozygous alleles in pools of captured target nucleic acids can be overcome.

Accordingly, in some embodiments, the disclosure provides methods that reduce variability in the detection of target nucleic acids in multiplex capture methods. In other embodiments, methods improve allelic representation in a capture pool and, thus, improve variant detection outcomes. In certain embodiments, the disclosure provides preparative methods for capturing target nucleic acids (e.g., genetic loci) that involve the use of different sets of multiple probes (e.g., molecular inversion probes MIPs) that capture overlapping regions of a target nucleic acid to achieve a more uniform representation of the target nucleic acids in a capture pool compared with methods of the prior art. In other embodiments, methods reduce bias, or the risk of bias, associated with large scale parallel capture of genetic loci, e.g., for diagnostic purposes. In other embodiments, methods are provided for increasing reproducibility (e.g., by reducing the effect of polymorphisms on target nucleic acid capture) in the detection of a plurality of genetic loci in parallel. In further embodiments, methods are provided for reducing the effect of probe synthesis and/or probe amplification variability on the analysis of a plurality of genetic loci in parallel.

According to some aspects, methods of analyzing a plurality of genetic loci are provided. In some embodiments, the methods comprise contacting each of a plurality of target nucleic acids with a probe set, wherein each probe set comprises a plurality of different probes, each probe having a central region flanked by a 5' region and a 3' region that are complementary to nucleic acids flanking the same strand of one of a plurality of subregions of the target nucleic acid, wherein the subregions of the target nucleic acid are different, and wherein each subregion overlaps with at least one other subregion, isolating a plurality of nucleic acids each having a nucleic acid sequence of a different subregion for each of the plurality of target nucleic acids, and analyzing the isolated nucleic acids.

In other embodiments, methods comprise contacting each of a plurality of target nucleic acids with a probe set, wherein each probe set comprises a plurality of different probes, each probe having a central region flanked by a 5' region and a 3' region that are complementary to nucleic acids flanking the same strand of one of a plurality of subregions of the target nucleic acid, wherein the subregions of the target nucleic acid are different, and wherein a portion of the 5' region and a portion of the 3' region of a probe have, respectively, the sequence of the 5' region and the sequence of the 3' region of a different probe, isolating a plurality of nucleic acids each having a nucleic acid sequence of a different subregion for each of the plurality of target nucleic acids, and analyzing the isolated nucleic acids.

In certain aspects, methods of the invention involve analyzing one or more genes with one or more molecular inversion probes provided in Appendix A. Particularly, those molecular inversion probes are used to capture various targets or subregions thereof on a gene selected from the group consisting of ABCC8, ASPA, BCKDHA, BCKDHB, BLM, CFTR, CLRN1, DLD, FANCC, G6PC, HEXA, IKBKAP, MCOLN1, PCDH15, and SMPD1. In certain applications, a set of two or more molecular inversion probes provided in Appendix A may be used to tile across different, but overlapping sub-regions of one or more genes so that one or more targets on the one or more genes are captured by at least two molecular inversion probes of the set. The number of molecular inversion probes used in a set for tile capture depends on the amount of overlapping coverage one desires for a certain target. In certain embodiments, a portion of one or more genes is captured using one or more molecular inversion probes in Appendix A. One or more molecular inversion probes of Appendix A may also be chosen to capture particular regions of interest, such as coding or noncoding regions, of a gene. In addition, one or more molecular inversion probes may be chosen to capture regions specific to certain diseases. The diseases may include, for example, Familial hyperinsulinism, Canavan disease, Maple syrup urine disease type **1***a*/**1***b*, Bloom syndrome, Cystic fibrosis, Usher syndrome type IIIA, Dihydrolipoamide dehydrogenase deficiency, Fanconi anemia group C, Glycogen storage disease type **1***a*, Tay-Sachs disease, Familial dysautonomia, Mucolipidosis type IV, Usher syndrome type IF, Niemann-Pick disease type A/B.

Aspects of the disclosure are based, in part, on the discovery of methods for overcoming problems associated with systematic and random errors (bias) in genome capture, amplification and sequencing methods, namely high variability in the capture and amplification of nucleic acids and disproportionate representation of heterozygous alleles in sequencing libraries.

Accordingly, in some embodiments, the disclosure provides methods that reduce errors associated with the variability in the capture and amplification of nucleic acids. In other embodiments, the methods improve allelic representation in sequencing libraries and, thus, improve variant detection outcomes. In certain embodiments, the disclosure provides preparative methods for capturing target nucleic acids (e.g., genetic loci) that involve the use of differentiator tag sequences to uniquely tag individual nucleic acid molecules. In some embodiments, the differentiator tag sequence permit the detection of bias based on the occurrence of combinations of differentiator tag and target sequences observed in a sequencing reaction. In other embodiments, the methods reduce errors caused by bias, or the risk of bias, associated with the capture, amplification and sequencing of genetic loci, e.g., for diagnostic purposes.

Aspects of the invention relate to providing sequence tags (referred to as differentiator tags) that are useful to determine whether target nucleic acid sequences identified in an assay are from independently isolated target nucleic acids or from multiple copies of the same target nucleic acid molecule (e.g., due to bias in a preparative step, for example, amplification). This information can be used to help analyze a threshold number of independently isolated target nucleic acids from a biological sample in order to obtain sequence information that is reliable and can be used to make a genotype conclusion (e.g., call) with a desired degree of confidence. This information also can be used to detect bias in one or more nucleic acid preparative steps.

In some embodiments, the methods disclosed herein are useful for any application where reduction of bias, e.g., associated with genomic isolation, amplification, sequencing, is important. For example, detection of cancer mutations in a heterogeneous tissue sample, detection of mutations in maternally-circulating fetal DNA, and detection of mutations in cells isolated during a preimplantation genetic diagnostic procedure.

Accordingly, in some aspects, methods of genotyping a subject are provided. In some embodiments, the methods comprise determining the sequence of at least a threshold number of independently isolated nucleic acids, wherein the sequence of each isolated nucleic acid comprises a target nucleic acid sequence and a differentiator tag sequence, wherein the threshold number is a number of unique combinations of target nucleic acid and differentiator tag sequences, wherein the isolated nucleic acids are identified as independently isolated if they comprise unique combinations of target nucleic acid and differentiator tag sequences, and wherein the target nucleic acid sequence is the sequence of a genomic locus of a subject.

In some embodiments, the isolated nucleic acids are products of a circularization selection-based preparative method, e.g., molecular inversion probe capture products. In other embodiments, the isolated nucleic acids are products of an amplification-based preparative methods. In other embodiments, the isolated nucleic acids are products of hybridization-based preparative methods.

Circularization selection-based preparative methods selectively convert regions of interest (target nucleic acids) into a covalently-closed circular molecule which is then isolated typically by removal (usually enzymatic, e.g. with exonuclease) of any non-circularized linear nucleic acid. Oligonucleotide probes (e.g., molecular inversion probes) are designed which have ends that flank the region of interest (target nucleic acid) and, optionally, primer sites, e.g., sequencing primer sites. The probes are allowed to hybridize to the genomic target, and enzymes are used to first (optionally) fill in any gap between probe ends and second ligate the probe closed. Following circularization, any remaining (non-target) linear nucleic acid is typically removed, resulting in isolation (capture) of target nucleic acid. Circularization selection-based preparative methods include molecular inversion probe capture reactions and 'selector' capture reactions. In some embodiments, molecular inversion probe capture of a target nucleic acid is indicative of the presence of a polymorphism in the target nucleic acid.

In amplification-based (e.g., PCR-based or LCR-based, etc.) preparative methods, genomic loci (target nucleic acids) are isolated directly by means of a polymerase chain reaction or ligase chain reaction (or other amplification method) that selectively amplifies each locus using one or more oligonucleotide primers. It is to be understood that primers will be sufficiently complementary to the target sequence to hybridize with and prime amplification of the target nucleic acid. Any one of a variety of art known methods may be utilized for primer design and synthesis. One or more of the primers may be perfectly complementary to the target sequence. Degenerate primers may also be used. Primers may also include additional nucleic acids that are not complementary to target sequences but that facilitate downstream applications, including for example restriction sites and differentiator tag sequences. Amplification-based methods include amplification of a single target nucleic acid and multiplex amplification (amplification of multiple target nucleic acids in parallel).

Hybridization-based preparative methods involve selectively immobilizing target nucleic acids for further manipulation. It is to be understood that one or more oligonucleotides (immobilization oligonucleotides), which comprise differentiator tag sequences, and which may be from 15 to 170 nucleotides in length, are used which hybridize along the length of a target region of a genetic locus to immobilize it. In some embodiments, immobilization oligonucleotides, are either immobilized before hybridization is performed (e.g., Roche/Nimblegen 'sequence capture'), or are prepared such that they include a moiety (e.g. biotin) which can be used to selectively immobilize the target nucleic acid after hybridization by binding to e.g., streptavidin-coated microbeads (e.g. Agilent 'SureSelect').

It should be appreciated that any of the circularization, amplification, and/or hybridization based methods described herein may be used in connection with one or more of the tiling/staggering, tagging, size-detection, and/or sensitivity enhancing algorithms described herein.

In some embodiments, the methods disclosed herein comprise determining the sequence of molecular inversion probe capture products, each comprising a molecular inversion probe and a target nucleic acid, wherein the sequence of the molecular inversion probe comprises a differentiator tag sequence and, optionally, a primer sequence, and wherein the target nucleic acid is a captured genomic locus of a subject, and genotyping the subject at the captured genomic locus based on the sequence of at least a threshold number of unique combinations of target nucleic acid and differentiator tag sequences of molecular inversion probe capture products.

In some embodiments, the methods disclosed herein comprise obtaining molecular inversion probe capture products, each comprising a molecular inversion probe and a target nucleic acid, wherein the sequence of the molecular inversion probe comprises a differentiator tag sequence and, optionally, a primer sequence, wherein the target nucleic acid is a captured genomic locus of the subject, amplifying the molecular inversion probe capture products, and genotyping the subject by determining, for each target nucleic acid, the sequence of at least a threshold number of unique combinations of target nucleic acid and differentiator tag sequence of molecular inversion probe capture products. In certain embodiments, obtaining comprises capturing target nucleic acids from a genomic sample of the subject with molecular inversion probes, each comprising a unique differentiator tag sequence. In specific embodiments, capturing is performed under conditions wherein the likelihood of obtaining two or more molecular inversion probe capture products with identical combinations of target and differentiator tag sequences is equal to or less than a predetermined value, optionally wherein the predetermined value is about 0.05.

In one embodiment, the threshold number for a specific target nucleic acid sequence is selected based on a desired statistical confidence for the genotype. In some embodiments, the methods further comprising determining a statistical confidence for the genotype based on the number of unique combinations of target nucleic acid and differentiator tag sequences.

According to some aspects, methods of analyzing a plurality of genetic loci are provided. In some embodiments, the methods comprise obtaining a plurality of molecular inversion probe capture products each comprising a molecular inversion probe and a target nucleic acid, wherein the sequence of the molecular inversion probe comprises a differentiator tag sequence and, optionally, a primer sequence (e.g., a sequence that is complementary to the sequence of a nucleic acid that is used as a primer for sequencing or other extension reaction), amplifying the plurality of molecular inversion probe capture products, determining numbers of occurrence of combinations of target nucleic acid and differentiator tag sequence of molecular inversion probe capture products in the amplified plurality, and if the number of occurrence of a specific combination of target nucleic acid sequence and differentiator tag sequence exceeds a predetermined value, detecting bias in the amplification of the molecular inversion probe comprising the specific combination. In some embodiments, the methods further comprise genotyping target sequences in the plurality, wherein the genotyping comprises correcting for bias, if detected.

In some embodiments, the target nucleic acid is a gene (or portion thereof) selected from Table 1. In some embodiments, the genotyping comprises determining the sequence of a target nucleic acid (e.g., a polymorphic sequence) at one or more (both) alleles of a genome (a diploid genome) of a subject. In certain embodiments, the genotyping comprises determining the sequence of a target nucleic acid at both alleles of a diploid genome of a subject, wherein in the target nucleic acid comprises, or consists of, a sequence of Table 1, Table 2, or other locus of interest.

In some embodiments, aspects of the invention provide methods and compositions for identifying nucleic acid insertions or deletions in genomic regions of interest without determining the nucleotide sequences of these regions. Aspects of the invention are particularly useful for detecting nucleic acid insertions or deletions in genomic regions containing nucleic acid sequence repeats (e.g., di- or trinucleotide repeats). However, the invention is not limited to analyzing nucleic acid repeats and may be used to detect insertions or deletions in any target nucleic acid of interest. Aspects of the invention are particularly useful for analyzing multiple loci in a multiplex assay.

In some embodiments, aspects of the invention relate to determining whether an amount of target nucleic acid that is captured in a genomic capture assay is higher or lower than expected. In some embodiments, a statistically significant deviation from an expected amount (e.g., higher or lower) is indicative of the presence of a nucleic acid insertion or deletion in the genomic region of interest. In some embodiments, the amount is a number of nucleic acid molecules that are captured. In some embodiments, the amount is a number of independently captured nucleic acid molecules in a sample. It should be appreciated that the captured nucleic acids may be literally captured from a sample, or their sequences may be captured without actually capturing the original nucleic acids in the sample. For example, nucleic acid sequences may be captured in an assay that involves a template-based extension of nucleic acids having the region of interest, in the sample.

Aspects of the invention are based on the recognition that the efficiency of certain capture techniques is affected by the length of the nucleic acid being captured. Accordingly, an increase or decrease in the length of a target nucleic acid (e.g., due to an insertion or deletion of a repeated sequence) can alter the capture efficiency of that nucleic acid. In some embodiments, a difference in the capture efficiency (e.g., a statistically significant difference in the capture efficiency) of a target nucleic acid is indicative of an insertion or deletion in the target nucleic acid. It should be appreciated that the capture efficiency for a target nucleic acid may be evaluated based on an amount of captured nucleic acid (e.g., number of captured nucleic acid molecules) relative to a control amount (e.g., based on an amount of control nucleic acid that is captured). However, the invention is not limited in this respect and other techniques for evaluating capture efficiency also may be used.

According to aspects of the invention, evaluating the capture efficiency as opposed to determining the sequence of the entire repeat region reduces errors associated with sequencing through repeat regions. Repeat sequences often give rise to stutters or skips in sequencing reactions that make it very difficult to accurately determine the number of repeats in a target region without running multiple sequencing reactions under different conditions and carefully analyzing the results. Such procedures are cumbersome and not readily scalable in a manner that is consistent with high throughput analyses of target nucleic acids. In some embodiments, repeat regions may be longer than the length of the individual sequence read, making length determination on the basis of a single read impossible. For example, when using next-generation sequencing the repeat regions may be longer than the length of the individual sequence read, making length determination on the basis of a single read impossible. Accordingly, aspects of the invention are useful to increase the sensitivity of detecting insertions or deletions in target regions, particularly target regions containing repeated sequences.

In some embodiments, aspects of the invention relate to capturing genomic nucleic acid sequences using a molecular inversion probe (e.g., MIP or Padlock probe) technique, and determining whether the amount (e.g., number) of captured sequences is higher or lower than expected. In some embodiments, the amount (e.g., number) of captured sequences is compared to an amount (e.g., number) of sequences captured in a control assay. The control assay may involve analyzing a control sample that contains a nucleic acid from the same genetic locus having a known sequence length (e.g., a known number of nucleic acid repeats). However, a control may involve analyzing a second (e.g., different) genetic locus that is not expected to contain any insertions or deletions. The second genetic locus may be analyzed in the same sample as the locus being interrogated or in a different sample where its length has been previously determined. The second genetic locus may be a locus that is not characterized by the presence of nucleic acid repeats (and thus not expected to contain insertions or deletions of the repeat sequence).

In some embodiments, a target nucleic acid region that is being evaluated may be determined by the identity of the targeting arms of a probe that is designed to capture the target region (or sequence thereof). For example, the targeting arms of a MIP probe may be designed to be complementary (e.g., sufficiently complementary for selective hybridization and/or polymerase extension and/or ligation) to genomic regions flanking a target region suspected of containing an insertion or deletion. It should be appreciated that two targeting arms may be designed to be complementary (e.g., sufficiently complementary for selective hybridization and/or polymerase extension and/or ligation) to the two flanking regions that are immediately adjacent (e.g., immediately 5' and 3', respectively) to a region of a sequence repeat on one strand of a genomic nucleic acid. However, one or both targeting arms may be designed to hybridize several bases (e.g., 1-5, 5-10, 10-25, 25-50, or more) upstream or downstream from the repeat region in such a way that the captured sequence includes a region of unique genomic sequence that on one or both sides of the repeat region. This unique region can then be used to identify the captured target (e.g., based on sequence or hybridization information).

In some embodiments, two or more (e.g., 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) different loci may be interrogated in parallel in a single assay (e.g., in a multiplex assay). In some embodiments, the ratio of captured nucleic acids for each locus may be used to determine whether a nucleic acid insertion or deletion is present in one locus relative to the other. For example, the ratio may be compared to a control ratio that is representative of the two loci when neither one has an insertion or deletion relative to control sequences (e.g., sequences that are normal or known to be associated with healthy phenotypes for those loci). However, the amount of captured nucleic acids may be compared to any suitable control as discussed herein.

The locus of a captured sequence may be identified by determining a portion of unique sequence 5' and/or 3' to the repeat region in the target nucleic acid suspected of containing a deletion or insertion. This does not require sequencing the captured repeat region itself. However, some or all of the repeat region also could be sequenced as aspects of the invention are not limited in this respect.

Aspects of the invention may be combined with one or more sequence-based assays (e.g., SNP detection assays), for example in a multiplex format, to determine the genotype of one or more regions of a subject.

In some embodiments, methods of detecting a polymorphism in a nucleic acid in a biological sample are provided. In some embodiments, the methods comprise evaluating the efficiency of capture at one or more loci and determining whether one or both alleles at that locus contain an insertion or deletion relative to a control locus (e.g., a locus indicative of a length of repeat sequence that is associated with a healthy phenotype).

Accordingly, aspects of the invention relate to methods for determining whether a target nucleic acid has an abnormal length by evaluating the capture efficiency of a target nucleic acid in a biological sample from a subject, wherein a capture efficiency that is different from a reference capture efficiency is indicative of the presence, in the biological sample, of a target nucleic acid having an abnormal length. It should be appreciated that the term “abnormal” is a relative term based on a comparison to a “normal” length. In some embodiments, a normal length is a length that is associated with a normal (e.g., healthy or non-carrier phenotype).

Accordingly, an abnormal length is a length that is either shorter or longer than the normal length. In some embodiments, the presence of an abnormal length is indicative of an increased risk that the locus is associated with a disease or a disease carrier phenotype. In some embodiments, the abnormal length is indicative that the subject is either has a disease or condition or is a carrier of a disease or condition (e.g., associated with the locus). However, it should be appreciated that the description of embodiments relating to detecting the presence of an abnormal length also support detecting the presence of a length that is different from an expected or control length.

In some embodiments, aspects of the invention relate to estimating the length of a target nucleic acid (e.g., of a sub-target region within a target nucleic acid). In some embodiments, aspects of the invention relate to methods for estimating the length of a target nucleic acid by contacting the target nucleic acid with a plurality of detection probes under conditions that permit hybridization of the detection probes to the target nucleic acid, wherein each detection probe is a polynucleotide that comprises a first arm that hybridizes to a first region of the target nucleic acid and a second arm that hybridizes to a second region of the target nucleic acid, wherein the first and second regions are on a common strand of the target nucleic acid, and wherein the nucleotide sequence of the target between the 5' end of the first region and the 3' end of the second region is the nucleotide sequence of a sub-target nucleic acid; and capturing a plurality of sub-target nucleic acids that are hybridized with the plurality of detection probes; and measuring the frequency of occurrence of a sub-target nucleic acid in the plurality of sub-target nucleic acids, wherein the frequency of occurrence of the sub-target nucleic acid in the plurality of sub-target nucleic acids is indicative of the length of the sub-target nucleic acid. It should be appreciated that methods for estimating a nucleic acid length may involve comparing a capture efficiency for a target nucleic acid region to two or more reference efficiencies for known nucleic acid lengths in order to determine whether the target nucleic acid region is smaller, intermediate, or larger in size than the known control lengths. In some embodiments, a series of nucleic acids of known different lengths may be used to provide a calibration curve for evaluating the length of a target nucleic acid region of interest.

In some embodiments, the capture efficiency of a target region suspected of having a deletion or insertion is determined by comparing the capture efficiency to a reference indicative of a normal capture efficiency. In some embodiments, the capture efficiency is lower than the reference capture efficiency. In some embodiments, the subject is identified as having an insertion in the target region. In some embodiments, the capture efficiency is higher than the reference capture efficiency. In some embodiments, the subject is identified as having a deletion in the target region. In some embodiments, the subject is identified as being heterozygous for the insertion. In some embodiments, the subject is identified as being heterozygous for the deletion.

In some embodiments of any of the methods described herein (e.g., tiling/staggering, tagging, size-detection, and/or sensitivity enhancement) aspects of the invention relate to capturing a sub-target nucleic acid (or a sequence of a sub-target nucleic acid). In some embodiments, a molecular inversion probe technique is used. In some embodiments, a molecular inversion probe is a single linear strand of nucleic acid that comprises a first targeting arm at its 5' end and a second targeting arm at its 3' end, wherein the first targeting arm is capable of specifically hybridizing to a first region flanking one end of the sub-target nucleic acid, and wherein the second targeting arm is capable of specifically hybridizing to a second region flanking the other end of the sub-target nucleic acid on the same strand of the target nucleic acid. In some embodiments, the first and second targeting arms are between about 10 and about 100 nucleotides long. In some embodiments, the first and second targeting arms are about 10-20, 20-30, 30-40, or 40-50 nucleotides long. In some embodiments, the first and second targeting arms are about 20 nucleotides long. In some embodiments, the first and second targeting arms have the same length. In some embodiments, the first and second targeting arms have different lengths. In some embodiments, each pair of first and second targeting arms in a set of probes has the same length. Accordingly, if one of the targeting arms is longer, the other one is correspondingly shorter. This allows for a quality control step in some embodiments to confirm that all captured probe/target sequence products have the same length after a multiplexed plurality of capture reactions. In some embodiments, a set of probes may be designed to have the same length if the intervening region is varied to accommodate any differences in the length of either one or both of the first and second targeting arms.

In some embodiments, the hybridization Tins of the first and second targeting arms are similar. In some embodiments, the hybridization Tms of the first and second targeting arms are within 2-5° C. of each other. In some embodiments, the hybridization Tms of the first and second targeting arms are identical. In some embodiments, the hybridization Tms of the first and second targeting arms are close to empirically-determined optima but not necessarily identical.

In some embodiments, the first and second targeting arms of a molecular inversion probe have different Tms. For example, the Tm of the first targeting arm (at the 5' end of the molecular inversion probe) may be higher than the Tm of the second targeting arm (at the 3' end of the molecular inversion probe). According to aspects of the invention, and without wishing to be bound by theory, a relatively high Tm for the first targeting arm may help avoid or prevent the first targeting arm from being displaced after hybridization by the extension product of the 3' end of the second targeting arm. It should be appreciated that a reference to the Tm of a targeting arm as used herein relates to the Tm of hybridization of the targeting arm to a nucleic acid having the complementary sequence (e.g., the region of the target nucleic acid that has a sequence that is complementary to the sequence of the targeting arm). It also should be appreciated that the Tms of the targeting arms described herein may be calculated using any appropriate method. For example, in some embodiments an experimental method (e.g., a gel shift assay, a hybridization assay, a melting curve analysis, for example in a PCR machine with a SYBR dye by stepping through a temperature ramp while monitoring signal level from an intercalating dye, for example, bound to a double-stranded DNA, etc.) may be used to determine one or more Tms empirically. In some embodiments, an optimal Tm may be determined by evaluating the number of products formed (e.g., for each of a plurality of MIP probes), and determining the optimal Tm as the center point in a histogram of Tm for all targeting arms. In some embodiments, a predictive algorithm may be used to determine a Tm theoretically. In some embodiments, a relatively simple predictive algorithm may be used based on the number of G/C and A/T base pairs when the sequence is hybridized to its target and/or the length of the hybridized product (e.g., for example, 64.9+41*([G+C]−16.4)/(A+T+G+C), see for example, Wallace, R. B., Shaffer, J., Murphy, R. F., Bonner, J., Hirose, T., and Itakura, K. (1979) Nucleic Acids Res 6:3543-3557). In some embodiments, a more complex algorithm may be used to account for the effects of base stacking entropy and enthalpy, ion concentration, and primer concentration (see, for example, SantaLucia J (1998), Proc Natl Acad Sci USA, 95:1460-5). In some embodiments an algorithm may use modified parameters (e.g., nearest-neighbor parameters for basepair entropy/enthalpy values). It should be appreciated that any suitable algorithm may be used as aspects of the invention are not limited in this respect. However, it also should be appreciated that different methodologies may results in different calculated or predicted Tins for the same sequences. Accordingly, in some embodiments, the same empirical and/or theoretical method is used to determine the Tins of different sequences for a set of probes to avoid a negative impact of any systematic difference in the Tm determination or prediction when designing a set of probes with predetermined similarities or differences for different Tins.

In some embodiments, the Tm of the first targeting arm may be about 1° C., about 2° C., about 3° C., about 4° C., about 5° C., or more than about 5° C. higher than the Tm of the second targeting arm. In some embodiments, each probe in a plurality of probes (e.g., each probe in a set of 5-10, each probe in a set of at least 10, each probe in a set of 10-50, each probe in a set of 50-100, each probe in a set of 100-500, each probe in a set of 500-1,000, each probe in a set of 1,000-1,500, each probe in a set of 1,500-2,000, each probe in a set of 2,000-3,000, 3,000-5,000, 5,000-10,000 or each probe in a set of at least 5,000 different probes) has a unique first targeting arm (e.g., they all have different sequences) and a unique second targeting arm (e.g., they all have different sequences). In some embodiments, for at least 10% of the probes (e.g., at least 25%, 25%-50%, 50%-75%, 75%-90%, 90%-95% or over 95%, or all of the probes) the first targeting arm has a Tm for its complementary sequence that is higher (e.g., about 1° C., about 2° C., about 3° C., about 4° C., about 5° C., or more than about 5° C. higher) than the Tm of the second targeting arm for its complementary sequence. In some embodiments, each of the first targeting arms have similar or identical Tins for their respective complementary sequences and each of the second targeting arms have similar or identical Tins for their respective complementary sequences (and the first targeting arms have higher Tins than the second targeting arms). For example, in some embodiments, the Tm of the first arm(s) may be about 58° C. and the Tm of the second arm(s) may be about 56° C. In some embodiments, the Tm of the first arm(s) may be about 68° C., and the Tm of the second arm(s) may be about 65° C. It should be appreciated that in some embodiments the similarity (e.g., within a range of 1° C., 2° C., 3° C., 4° C., 5° C.) or identity of the Tins for the different targeting arms should be based either on empirical data for each arm or based on the same predictive algorithm for each arm (e.g., Wallace, R. B., Shaffer, J., Murphy, R. F., Bonner, J., Hirose, T., and Itakura, K. (1979) Nucleic Acids Res 6:3543-3557, SantaLucia J (1998), Proc Natl Acad Sci USA, 95:1460-5, or other algorithm).

In some embodiments, the Tm of the first targeting arm of a molecular inversion probe (at the 5' end of the molecular inversion probe) is selected to be sufficiently stable to prevent displacement of the first targeting arm from its complementary sequence on a target nucleic acid. In some embodiments, the Tm of the first targeting arm is 50-55° C., at least 55° C., 55-60° C., at least 60° C., 60-65° C., at least 65° C., at least 70° C., at least 75° C., or at least 80° C. As discussed above, it should be appreciated that the for a particular targeting arm may be determined empirically or theoretically. Different theoretical models may be used to determine a Tm and it should be appreciated that the predicted Tm for a particular sequence may be different depending on the algorithm used for the prediction. In some embodiments, each probe in a plurality of probes (e.g., each probe in a set of 5-10, each probe in a set of at least 10, each probe in a set of 10-50, each probe in a set of 50-100, each probe in a set of 100-500, or each probe in a set of at least 500 different probes) has a different first targeting arm (e.g., different sequences) but each different first targeting arm has a similar or identical Tm for its complementary sequence on a target nucleic acid. It should be appreciated that in some embodiments the similarity (e.g., within a range of 1 C, 2 C, 3 C, 4 C, 5 C) or identity of the Tms for the different targeting arms should be based either on empirical data for each arm or based on the same predictive algorithm for each arm (e.g., Wallace, R. B., Shaffer, J., Murphy, R. F., Bonner, J., Hirose, T., and Itakura, K. (1979) Nucleic Acids Res 6:3543-3557, SantaLucia J (1998), Proc Natl Acad Sci USA, 95:1460-5, or other algorithm).

In some embodiments, the sub-target nucleic acid contains a nucleic acid repeat. In some embodiments, the nucleic acid repeat is a dinucleotide or trinucleotide repeat. In some embodiments, the sub-target nucleic acid contains 10-100 copies of the nucleic acid repeat in the absence of an abnormal increase or decrease in nucleic acid repeats. In some embodiments, the sub-target nucleic acid is a region of the Fragile-X locus that contains a nucleic acid repeat. In some embodiments, one or both targeting arms hybridize to a region on the target nucleic acid that is immediately adjacent to a region of nucleic acid repeats. In some embodiments, one or both targeting arms hybridize to a region on the target nucleic acid that is separated from a region of nucleic acid repeats by a region that does not contain any nucleic acid repeats. In some embodiments, the molecular inversion probe further comprises a primer-binding region that can be used to sequence the captured sub-target nucleic acid and optionally the first and/or second targeting arm.

In some embodiments, aspects of the invention relate to evaluating the length of a plurality of different target nucleic acids in a biological sample. In some embodiments, the plurality of target nucleic acids are analyzed using a plurality of different molecular inversion probes. In some embodiments, each different molecular inversion probe comprises a different pair of first and second targeting arms at each of the 3' and 5' ends. In some embodiments, each different molecular inversion probe comprises the same primer-binding sequence.

In some embodiments, aspects of the invention relate to analyzing nucleic acid from a biological sample obtained from a subject. In some embodiments, the biological sample is a blood sample. In some embodiments, the biological sample is a tissue sample, specific cell population, tumor sample, circulating tumor cells, or environmental sample. In some embodiments, the biological sample is a single cell. In some embodiments, nucleic acids are analyzed in biological samples obtained from a plurality of different subjects. In some embodiments, nucleic acids from a biological sample are analyzed in multiplex reactions. It should be appreciated that a biological sample contains a plurality of copies of a genome derived from a plurality of cells in the sample. Accordingly, a sample may contain a plurality of independent copies of a target nucleic acid region of interest, the capture efficiency of which can be used to evaluate its size as described herein.

In some embodiments, aspects of the invention relate to evaluating a nucleic acid capture efficiency by determining an amount of target nucleic acid that is captured (e.g., an amount of sub-target nucleic acid sequences that are captured). In some embodiments, the amount of target nucleic acid that is captured is determined by determining a number of independently captured target nucleic acid molecules (e.g., the amount of independently captured molecules that have the sequence of the sub-target region). In some embodiments, the amount of target nucleic acid that is captured is compared to a reference amount of captured nucleic acid. In some embodiments, the reference amount is determined by determining a number of independently captured molecules of a reference nucleic acid. In some embodiments, the reference nucleic acid is a nucleic acid of a different locus in the biological sample that is not suspected of containing a deletion or insertion. In some embodiments, the reference nucleic acid is a nucleic acid of known size and amount that is added to the capture reaction. As described herein, a number of independently captured nucleic acid sequences can be determined by contacting a nucleic acid sample with a preparation of a probe (e.g., a MIP probe as described herein). It should be appreciated that the preparation may comprise a plurality of copies of the same probe and accordingly a plurality of independent copies of the target region may be captured by different probe molecules. The number of probe molecules that actually capture a sequence can be evaluated by determining an amount or number of captured molecules using any suitable technique. This number is a reflection of both the number of target molecules in the sample and the efficiency of capture of those target molecules, which in turn is related to the size of the target molecules as described herein. Accordingly, the capture efficiency can be evaluated by controlling for the abundance of the target nucleic acid, for example by comparing the number or amount of captured target molecules to an appropriate control (e.g., a known size and amount of control nucleic acid, or a different locus that should be present in the same amount in the biological sample and is not expected to contain any insertions or deletions). It should be appreciated that other factors may affect the capture efficiency of a particular target nucleic acid region (e.g., the sequence of the region, the GC content, the presence of secondary structures, etc.). However, these factors also can be accounted for by using appropriate controls (e.g., known sequences having similar properties, the same sequences, other genomic sequences expected to be present in the biological sample at the same frequency, etc., or any combination thereof).

In some embodiments, aspects of the invention relate to identifying a subject as having an insertion or deletion in one or more alleles of a genetic locus if the capture efficiency for that genetic locus is statistically significantly different than a reference capture efficiency. It should be appreciated that hybridization conditions used for any of the capture techniques described herein (e.g., MIP capture techniques) can be based on known hybridization buffers and conditions.

In some embodiments, the methods disclosed herein are useful for any application where the detection of deletions or insertions is important.

In some embodiments, aspects of the invention relate to basing a nucleic acid sequence analysis on results from two or more different nucleic acid preparatory techniques that have different systematic biases in the types of nucleic acids that they sample. According to the invention, different techniques have different sequence biases that are systematic and not simply due to stochastic effects during nucleic acid capture or amplification. Accordingly, the degree of oversampling required to overcome variations in nucleic acid preparation needs to be sufficient to overcome the biases (e.g., an oversampling of 2-5 fold, 5-10 fold, 5-15 fold, 15-20 fold, 20-30 fold, 30-50 fold, or intermediate to higher fold).

According to some embodiments, different techniques have different characteristic or systematic biases. For example, one technique may bias a sample analysis towards one particular allele at a genetic locus of interest, whereas a different technique would bias the sample analysis towards a different allele at the same locus. Accordingly, the same sample may be identified as being different depending on the type of technique that is used to prepare nucleic acid for sequence analysis. This effectively represents a sensitivity limitation, because each technique has different relative sensitivities for polymorphic sequences of interest.

According to aspects of the invention, the sensitivity of a nucleic acid analysis can be increased by combining the sequences from different nucleic acid preparative steps and using the combined sequence information for a diagnostic assay (e.g., for a making a call as to whether a subject is homozygous or heterozygous at a genetic locus of interest).

In some embodiments, the invention provides a method of increasing the sensitivity of a nucleic acid detection assay by obtaining a first preparation of a target to nucleic acid using a first preparative method on a biological sample, obtaining a second preparation of a target nucleic acid using a second preparative method on the biological sample, assaying the sequences obtained in both first and second nucleic acid preparations, and using the sequence information from both first and second nucleic acid preparations to determine the genotype of the target nucleic acid in the biological sample, wherein the first and second preparative methods have different systematic sequence biases. In some embodiments, the first and second nucleic acid preparations are combined prior to performing a sequence assay. In some embodiments, separate sequence assays are performed on the first and second nucleic acid preparations and the sequence information from both assays are combined to determine the genotype of the target nucleic acid in the biological sample. In some embodiments, the first preparative method is an amplification-based, a hybridization-based, or a circular probe-based preparative method. In some embodiments, the second method is an amplification-based, a hybridization-based, or a circular probe-based preparative method. In some embodiments, the first and second methods are of different types (e.g., only one of them is an amplification-based, a hybridization-based, or a circular probe-based preparative method, and the other one is one or the other two types of method). Accordingly, in some embodiments the second preparative method is an amplification-based, a hybridization-based, or a circular probe-based preparative method, provided that the second method is different from the first method. However, in some embodiments, both methods may be of the same type, provided they are different methods (e.g., both are amplification based or hybridization-based, but are different types of amplification or hybridization methods, e.g., with different relative biases).

In amplification-based (e.g., PCR-based or LCR-based, etc.) preparative methods, genomic loci (target nucleic acids) are isolated directly by means of a polymerase chain reaction or ligase chain reaction (or other amplification method) that selectively amplifies each locus using a pair of oligonucleotide primers. It is to be understood that primers will be sufficiently complementary to the target sequence to hybridize with and prime amplification of the target nucleic acid. Any one of a variety of art known methods may be utilized for primer design and synthesis. One or both of the primers may be perfectly complementary to the target sequence.

Degenerate primers may also be used. Primers may also include additional nucleic acids that are not complementary to target sequences but that facilitate downstream applications, including for example restriction sites and identifier sequences (e.g., source sequences). PCR based methods may include amplification of a single target nucleic acid and multiplex amplification (amplification of multiple target nucleic acids in parallel).

Hybridization-based preparative may methods involve selectively immobilizing target nucleic acids for further manipulation. It is to be understood that one or more oligonucleotides (immobilization oligonucleotides), which in some embodiments may be from 10 to 200 nucleotides in length, are used which hybridize along the length of a target region of a genetic locus to immobilize it. In some embodiments, immobilization oligonucleotides are either immobilized before hybridization is performed (e.g., Roche/Nimblegen 'sequence capture'), or are prepared such that they include a moiety (e.g., biotin) which can be used to selectively immobilize the target nucleic acid after hybridization by binding to e.g., streptavidin-coated microbeads (e.g., Agilent 'SureSelect').

Circularization selection-based preparative methods selectively convert each region of interest into a covalently-closed circular molecule which is then isolated by removal (usually enzymatic, e.g., with exonuclease) of any non-circularized linear nucleic acid. Oligonucleotide probes are designed which have ends that flank the region of interest. The probes are allowed to hybridize to the genomic target, and enzymes are used to first (optionally) fill in any gap between probe ends and second ligate the probe closed. In some embodiments, following circularization, any remaining (non-target) linear nucleic acid can be removed, resulting in isolation (capture) of target nucleic acid. Circularization selection-based preparative methods include molecular inversion probe capture reactions and 'selector' capture reactions. However, other techniques may be used as aspects of the invention are not limited in this respect. In some embodiments, molecular inversion probe capture of a target nucleic acid is indicative of the presence of a polymorphism in the target nucleic acid.

A variety of methods may be used to evaluate and compare bias profiles of each preparative technique. Next-generation sequencing may be used to quantitatively measure the abundance of each isolated target nucleic acid obtained from a certain preparative method. This abundance may be compared to a control abundance value (e.g., a known starting abundance of the target nucleic acid) and/or with an abundance determined through the use of an alternative preparative method. For example, a set of target nucleic acids may be isolated by one or more of the three preparative methods; the target nucleic acid may be observed x times using the amplification technique, y times using the hybridization enrichment technique, and z times using the circularization selection technique. A pairwise correlation coefficient may be computed between each abundance value (e.g., x and y, x and z, and y and z) to assess bias in nucleic acid isolation between pairs of preparative methods. Since the mechanisms of isolation are different in each approach, the abundances will usually be different and largely uncorrelated with each other.

In some embodiments, the invention provides a method of obtaining a nucleic acid preparation that is representative of a target nucleic acid in a biological sample by obtaining a first preparation of a target nucleic acid using a first preparative method on a biological sample, obtaining a second preparation of a target nucleic acid using a second preparative method on the biological sample, and combining the first and second nucleic acid preparations to obtain a combined preparation that is representative of the target nucleic acid in the biological sample.

In some embodiments of any of the methods described herein, a third preparation of the target nucleic acid is obtained using a third preparative method that is different from the first and second preparative methods, wherein the first, second, and third preparative methods all have different systematic sequence biases. In some embodiments of any of the methods described herein, the different preparative methods are used for a plurality of different loci in the biological sample to increase the sensitivity of a multiplex nucleic acid analysis. In some embodiments, the target nucleic acid has a sequence of a gene selected from Table 1.

However, it should be appreciated that a genotyping method of the invention may include several steps, each of which independently may involve one or more different preparative techniques described herein. In some embodiments, a nucleic acid preparation may be obtained using one or more (e.g., 2, 3, 4, 5, or more) different techniques described herein (e.g., amplification, hybridization capture, circular probe capture, etc., or any combination thereof) and the nucleic acid preparation may be analyzed using one or more different techniques (e.g., amplification, hybridization capture, circular probe capture, etc., or any combination thereof) that are selected independently of the techniques used for the initial preparation.

In some embodiments, aspects of the invention also provide compositions, kits, devices, and analytical methods for increasing the sensitivity of nucleic acid assays. Aspects of the invention are particularly useful for increasing the confidence level of genotyping analyses. However, aspects of the invention may be used in the context of any suitable nucleic acid analysis, for example, but not limited to, a nucleic acid analysis that is designed to determine whether more than one sequence variant is present in a sample.

In some embodiments, aspects of the invention relate to a plurality of nucleic acid probes (e.g., 10-50, 50-100, 100-250, 250-500, 500-1,000, 1,000-2,000, 2,000-5,000, 5,000-7,500, 7,500-10,000, or lower, higher, or intermediate number of different probes). In some embodiments, each probe or each of a subset of probes (e.g., 10-25%, 25-50%, 50-75%, 75-90%, or 90-99%) has a different first targeting arm. In some embodiments, each probe or each probe of a subset of probes (e.g., 10-25%, 25-50%, 50-75%, 75-90%, or 90-99%) has a different second targeting arm. In some embodiments, the first and second targeting arms are separated by the same intervening sequence. In some embodiments, the first and second targeting arms are complementary to target nucleic acid sequences that are separated by the same or a similar length (e.g., number of nucleic acids, for example, 0-25, 25-50, 50-100, 100-250, 250-500, 500-1,000, 1,000-2,500 or longer or intermediate number of nucleotides) on their respective target nucleic acids (e.g., genomic loci). In some embodiments, each probe or a subset of probes (e.g., 10-25%, 25-50%, 50-75%, 75-90%, or 90-99%) includes a first primer binding sequence. In some embodiments, the primer binding sequence is the same (e.g., it can be used to prime sequencing or other extension reaction). In some embodiments, each probe or a subset of probes (e.g., 10-25%, 25-50%, 50-75%, 75-90%, or 90-99%) includes a unique identifier sequence tag (e.g., that is predetermined and can be used to distinguish each probe).

In some embodiments, the methods disclosed herein are useful for any application where sensitivity is important. For example, detection of cancer mutations in a heterogenous tissue sample, detection of mutations in maternally-circulating fetal DNA, and detection of mutations in cells isolated during a preimplantation genetic diagnostic procedure.

According to some aspects of the invention, methods of detecting a polymorphism in a nucleic acid in a biological sample are provided. In some embodiments, the methods comprise obtaining a nucleic acid preparation using a preparative method (e.g., any of the preparative methods disclosed herein) on a biological sample, and performing a molecular inversion probe capture reaction on the nucleic acid preparation, wherein a molecular inversion probe capture (e.g., using a mutation-detection MIP) of a target nucleic acid of the nucleic acid preparation is indicative of the presence of a mutation (polymorphism) in the target nucleic acid, optionally wherein the polymorphism is selected from Table 2.

According to some aspects of the invention, methods of genotyping a nucleic acid in a biological sample are provided. In some embodiments, the methods comprise obtaining a nucleic acid preparation using a preparative method on a biological sample, sequencing a target nucleic acid of the nucleic acid preparation, and performing a molecular inversion probe capture reaction on the biological sample, wherein a molecular inversion probe capture of the target nucleic acid in the biological sample is indicative of the presence of a polymorphism in the target nucleic acid, genotyping the target nucleic acid based on the results of the sequencing and the capture reaction.

In some embodiments of the methods disclosed herein, the target nucleic acid has a sequence of a gene selected from Table 1.

It should be appreciated that any one or more embodiments described herein may be used for evaluating multiple genetic markers in parallel. Accordingly, in some embodiments, aspects of the invention relate to determining the presence of one or more markers (e.g., one or more alleles) at multiple different genetic loci in parallel. Accordingly, the risk or presence of multiple heritable disorders may be evaluated in parallel. In some embodiments, the risk of having offspring with one or more heritable disorders may be evaluated. In some embodiments, an evaluation may be performed on a biological sample of a parent or a child (e.g., at a pre-implantation, prenatal, perinatal, or postnatal stage). In some embodiments, the disclosure provides methods for analyzing multiple genetic loci (e.g., a plurality of target nucleic acids selected from Table 1 or 2) from a patient sample, such as a blood, pre-implantation embryo, chorionic villus or amniotic fluid sample. A patient or subject may be a human. However, aspects of the invention are not limited to humans and may be applied to other species (e.g., mammals, birds, reptiles, other vertebrates or invertebrates) as aspects of the invention are not limited in this respect. A subject or patient may be male or female. In some embodiments, in connection with reproductive genetic counseling, samples from a male and female member of a couple may be analyzed. In some embodiments, for example, in connection with an animal breeding program, samples from a plurality of male and female subjects may be analyzed to determine compatible or optimal breeding partners or strategies for particular traits or to avoid one or more diseases or conditions. Accordingly, reproductive risks may be determined and/or reproductive recommendations may be provided based on information derived from one or more embodiments of the invention.

However, it should be appreciated that aspects of the invention may be used in connection with any medical evaluation where the presence of one or more alleles at a genetic locus of interest is relevant to a medical determination (e.g., risk or detection of disease, disease prognosis, therapy selection, therapy monitoring, etc.). Further aspects of the invention may be used in connection with detection, in tumor tissue or circulating tumor cells, of mutations in cellular pathways that cause cancer or predict efficacy of treatment regimens, or with detection and identification of pathogenic organisms in the environment or a sample obtained from a subject, e.g., a human subject.

These and other aspects of the invention are described in more detail in the following description and non-limiting examples and drawings.

BRIEF DESCRIPTION OF DRAWINGS

FIG. **1** illustrates a non-limiting embodiment of a tiled probe layout;

FIG. **2** illustrates a non-limiting embodiment of a staggered probe layout;

FIG. **3** illustrates a non-limiting embodiment of an alternating staggered probe layout;

FIG. **4**, panels a), b), and c) depict various non-limiting methods for combining differentiator tag sequence and target sequences (NNNN depicts a differentiator tag sequence);

FIG. **5** depicts a non-limiting method for genotyping based on target and differentiator tag sequences;

FIG. **6** depicts non-limiting results of a simulation of a MIP capture reaction; FIG. **7** depicts a non-limiting graph of sequencing coverage;

FIG. **8** illustrates that shorter sequences are captured with higher efficiency that longer sequences using MIPs;

FIG. **9** illustrates a non-limiting scheme of padlock (MIP) capture of a region that includes both repetitive regions (thick wavy line) and the adjacent unique sequence (thick strait line);

FIG. **10** illustrates a non-limiting hypothetical relationship between target gap size and the relative number of reads of the repetitive region;

FIG. **11**A depicts MIP capture of FMR1 repeat regions from a diploid genome;

FIG. **11**B depicts preparative methods for biallelic resolution of FMR1 repeat region lengths in a diploid genome using MIP capture probes and unique differentiator tags;

FIG. **11**C depicts an analysis of FMR1 repeat region lengths in a diploid genome;

FIG. **12** is a schematic of an embodiment of an algorithm of the invention;

FIG. **13** illustrates a non-limiting example of a graph of per-target abundance with MIP capture; and,

FIG. **14** shows a non-limiting a graph of correlation between two MIP capture reactions.

FIGS. **15**A-B show a SNaPshot validation of a putative Sanger variant call. FIG. **15**A discloses "GM1 7080" sequences as SEQ ID NO: 6328, 6329, and 6328 and FIG. **15**B discloses the "GM1 7074" sequences as SEQ ID NO: 6328, 6328, and 6328, all respectively, in order of appearance.

FIGS. **16**A-**16**D depict skewed allelic fractions in aneuploid cell line GM18540. FIG. **16**A depicts an IGV view of NGS data from GM18540 for the genotype call of interest (shown between vertical lines) (FIG. **16**A discloses SEQ ID NO: 6330-6331). FIG. **16**B depicts bi-directional Sanger data for the variant-containing region. FIG. **16**C depicts a histogram of allele ratios for all non-reference genotype calls in chromosome 11 derived from whole genome shotgun sequencing (WGSS) of GM18540 and control sample GM18537. FIG. **16**D depicts genome-wide relative coverage for GM18540. WGSS coverage data for each of the 25 autosomes was binned into 50 Kb intervals and the log-ratio of the per-sample mean normalized values was plotted versus chromosome position. Dashed vertical lines denote chromosome boundaries; within a chromosome the ratios are arranged according to genomic position.

FIGS. **17**A-D depict detection of previously-uncharacterized mutations in samples from individuals affected with cystic fibrosis. FIG. **17**A depicts IGV of heterozygous splice site mutation c.3368−2A>T in sample GM12960 (FIG. **17**A discloses SEQ ID NO: 6332-6333). FIG. **17**B depicts IGV of heterozygous premature stop codon mutation R1158X in sample GM18802 (FIG. **17**B discloses SEQ ID NO: 6334-

6335). FIG. **17**C depicts Sanger data confirming existence of mutation c.3368−2A>T in sample GM12960 (FIG. **17**C discloses SEQ ID NO: 6336 and 6336). FIG. **17**D depicts Sanger data confirming existence of mutation R1158X in sample GM18802 (FIG. **17**D discloses SEQ ID NO: 6337 and 6337).

FIGS. **18**A-E depict next-generation DNA sequencing workflow according to certain embodiments. FIG. **18**B discloses (top panel) SEQ ID NO: 6338-6349, (left panel) SEQ ID NO: 6338-6343, and (right panel) SEQ ID NO: 6344-6349, all respectively, in order of appearance. FIG. **18**C discloses SEQ ID NO: 6350-6356, 6353, 6352, 6357, and 6357, respectively, in order of appearance. FIG. **18**D discloses (left panel) SEQ ID NO: 6352, 6358, 6350, 6352, 6358, 6350, 6359, and 6359, and (right panel) SEQ ID NO: 6360, 6361, 6355, 6360, 6361, 6355, 6362, and 6363, all respectively, in order of appearance. FIG. **18**E discloses (left panel) SEQ ID NO: 6358, 6352, and 6350, (right panel) SEQ ID NO: 6360, 6361, and 6355, and (bottom panel) SEQ ID NO: 6364 and 6364, all respectively, in order of appearance.

FIGS. **19**A-D depict data from genotyping by assembly template alignment (GATA). GATA correctly genotypes insertions and deletions that are undetectable by the Alignment Only method. Read from top to bottom, each panel provides tracks for cumulative depth of coverage (vertical grey bars); representative MIP alignments (horizontal grey bars) with mismatches (letters), insertions (black bars), and gaps (dashed lines); chromatogram; reference DNA and amino acid sequence for FIG. **19**A heterozygous BLM c.2207_2212delinsTAGATTC in sample GM04408 as well as several alleles in the first exon of SMPD1 (FIG. **19**A discloses SEQ ID NO: 6365 and 6366) including FIG. **19**B a heterozygous 18 bp deletion in sample GM20342 (minus strand) (FIG. **19**B discloses SEQ ID NO: 6367 and 6368), FIG. **19**C a heterozygous 12 bp insertion and homozygous substitution in sample GM17282 (plus strand) (FIG. **19**C discloses SEQ ID NO: 6369 and 6370), and FIG. **19**D compound heterozygous 6 and 12 bp deletions in sample GM00502 (minus strand) (FIG. **19**D discloses SEQ ID NO: 6369 and 6370). Chromatogram trace offsets corresponding to specific heterozygous insertion and deletion patterns are indicated with slanted lines color coded by reference base. For clarity offsets are shown for FIGS. **19**C-D only.

FIGS. **20**A-**1**, **20**A-**2**, **20**A-**3**, **20**B-**1**, **20**B-**2** and **20**B-**3** show NGS detection of allele dropout in Sanger reactions. FIG. **20**A-**1** discloses SEQ ID NO: 6371, 6372, and 6372, FIG. **20**A-**2** depicts SEQ ID NO: 6371, 6371, 6372, and FIG. **20**A-**3** discloses SEQ ID NOS 6373; and 6374, all respectively, in order of appearance. FIG. **20**B-**1** discloses SEQ ID NO: 6371, 6372, and 6372, FIG. **20**B-**2** discloses SEQ ID NO: 6371, 6371, 6372, and FIG. **20**B-**3** discloses SEQ ID NO: 6373 and 6374, all respectively, in order of appearance.

FIG. **21** diagrams use of methods of the invention to validate a genotyping by assembly-templated alignment (GATA) technique.

FIG. **22** illustrates obtaining sequence reads and inserting a simulated mutation.

FIG. **23** shows standard analysis of sequence reads for comparison to GATA.

FIG. **24** shows analysis by GATA.

DETAILED DESCRIPTION

Aspects of the invention relate to preparative and analytical methods and compositions for evaluating genotypes, and in particular, for determining the allelic identity (or identities in a diploid organism) of one or more genetic loci in a subject. Aspects of the invention are based, in part, on the identification of different sources of ambiguity and error in genetic analyses, and, in part, on the identification of one or more approaches to avoid, reduce, recognize, and/or resolve these errors and ambiguities at different stages in a genetic analysis. Aspects of the invention relate to methods and compositions for addressing bias and/or stochastic variation associated with one or more preparative and/or analytical steps of a nucleic acid evaluation technology. In some embodiments, preparative methods can be adapted to avoid or reduce the risk of bias skewing the results of a genetic analysis. In some embodiments, analytical methods can be adapted to recognize and correct for data variations that may give rise to misinterpretation (e.g., incorrect calls such as homozygous when the subject is actually heterozygous or heterozygous when the subject is actually homozygous). Methods of the invention may be used for any type of mutation, for example a single base change (e.g., insertion, deletion, transversion or transition, etc.), a multiple base insertion, deletion, duplication, inversion, and/or any other change or combination thereof.

In some embodiments, additional or alternative techniques may be used to address loci characterized by multiple repeats of a core sequence where the length of the repeat is longer than a typical sequencing read thereby making it difficult to determine whether a deletion or duplication of one or more core sequence units has occurred based solely on a sequence read. In some embodiments, increased confidence in an assay result may be obtained by i) selecting two or more different preparative and/or analytical techniques that have different biases (e.g., known to have different biases), ii) evaluating a patient sample using the two or more different techniques, iii) comparing the results from the two or more different techniques, and/or iv) determining whether the results are consistent for the two or more different techniques. In some embodiments, if determining in step (iv) indicates that the results are consistent (e.g., the same) then increased confidence in the assay result is obtained. In other embodiments, if determining in step (iv) indicates that the results are inconsistent (e.g., that the results are ambiguous) then one or more additional preparative and/or analytical techniques, which have a different bias (e.g., known to have a different bias) compared with the two or more different preparative and/or analytical techniques selected in step (i), are used to evaluate the patient sample, and the results of the one or more additional preparative and/or analytical techniques are compared with the results from step (ii) to resolve the inconsistency.

In some embodiments, two or more independent samples may be obtained from a subject and independently analyzed. In some embodiments, two or more independent samples are obtained at approximately the same time point. In some embodiments, two or more independent samples are obtained at multiple different time points. In some embodiments, the use of two or more independent sample facilitates the elimination, normalization, and/or quantification of stochastic measurement noise. It is to be appreciated that two or more independent samples may be obtained in connection with any of the methods disclosed herein, including, for example, methods for pathogen profiling in a human or other animal subjects, monitoring tumor progression/regression, analyzing circulating tumor cells, analyzing fetal cells in maternal circulation, and analyzing/monitoring/profiling of environmental pathogens.

In some embodiments, one or more of the techniques described herein may be combined in a single assay protocol for evaluating multiple patient samples in parallel.

It should be appreciated that aspects of the invention may be useful for high throughput, cost-effective, yet reliable, genotyping of multiple patient samples (e.g., in parallel, for example in multiplex reactions). In some embodiments, aspects of the invention are useful to reduce the error frequency in a multiplex analysis. Certain embodiments may be particularly useful where multiple reactions (e.g., multiple loci and/or multiple patient samples) are being processed. For example, 10-25, 25-50, 50-75, 75-100 or more loci may be evaluated for each subject out of any number of subject samples that may be processed in parallel (e.g., 1-25, 25-50, 50-100, 100-500, 500-1,000, 1,000-2,500, 2,500-5,000 or more or intermediate numbers of patient samples). It should be appreciated that different embodiments of the invention may involve conducting two or more target capture reactions and/or two or more patient sample analyses in parallel in a single multiplex reaction. For example, in some embodiments a plurality of capture reactions (e.g., using different capture probes for different target loci) may be performed in a single multiplex reaction on a single patient sample. In some embodiments, a plurality of captured nucleic acids from each one of a plurality of patient samples may be combined in a single multiplex analysis reaction. In some embodiments, samples from different subjects are tagged with subject-specific (e.g., patient-specific) tags (e.g., unique sequence tags) so that the information from each product can be assigned to an identified subject. In some embodiments, each of the different capture probes used for each patient sample have a common patient-specific tag. In some embodiments, the capture probes do not have patient-specific tags, but the captured products from each subject may be amplified using one or a pair of amplification primers that are labeled with a patient-specific tag. Other techniques for associating a patient-specific tag with the captured product from a single patient sample may be used as aspects of the invention are not limited in this respect. It should be appreciated that patient-specific tags as used herein may refer to unique tags that are assigned to identified patients in a particular assay. The same tags may be used in a separate multiplex analysis with a different set of patient samples (e.g., from different patients) each of which is assigned one of the tags. In some embodiments, different sets of unique tags may be used in sequential (e.g., alternating) multiplex reactions in order to reduce the risk of contamination from one assay to the next and allow contamination to be detected on the basis of the presence of tags that are not expected to be present in a particular assay.

Embodiments of the invention may be used for any of a number of different settings: reproductive settings, disease screening, identifying subjects having cancer, identifying subjects having increased risk for a disease, stratifying a population of subjects according to one or more of a number of factors, for example responsiveness to a particular drug, lack or not of an adverse reaction (or risk therefore) to a particular drug, and/or providing information for medical records (e.g., homozygosity, heterozygosity at one or more loci). It should be appreciated that the invention is not limited to genomic analysis of patient samples. For example, aspects of the invention may be useful for high throughput genetic analysis of environment samples to detect pathogens.

In some embodiments, the methods disclosed herein are useful for diagnosis of one or more heritable disorders. In some embodiments, a heritable disorder that may be diagnosed with the methods disclosed herein is a genetic disorder that is prevalent in the Ashkenazi Jewish population. In some embodiments, the heritable disorders are selected from: 21-Hydroxylase-Deficient Congenital Adrenal Hyperplasia; ABCC8-Related Hyperinsulinism; Alpha-Thalassemia, includes Constant Spring, & MR associated; Arylsulfatase A Deficiency-Metyachromatic Leukodystrophy; Biotimidase Deficiency-Holocarboxylase Synthetase Deficiency; Bloom's Syndrome; Canavan Disease; CFTR-Related Disorders-cystic fibrosis; Citrullinemia Type I; Combined MMA & Homocystinuria-db1C; Dystrophinopathies (DMD & BMD); Familial Dysautonomia; Fanconi Anemia-FANCC; Galactosemia-C1-assical: Galactokinase Deficiency & Galactose Epimerase Deficiency; Gaucher Disease; GJB2-Related DFNB 1 Nonsyndromic Hearing Loss and Deafness; Glutaric acidemia Type 1; Hemoglobinopathies beta-chain disorders; Glycogen Storage Disease Type 1A; Maple Syrup Urine Disease; Types 1A, 1B, 2, 3; Medium Chain Acyl-Coenzyme A; Dehydrogenase Deficiency-MCADD; Methylmalonic Acidemia; Mucolipidosis IV; Nemaline Myopathy; Nieman-Pick Type A-Acid Sphingomyelinase Deficiency; Non-Ketotic Hyperglycinemia-Glycine Encephalopathy; Ornithine Transcarbamylase Deficiency; PKU Phenylalanine Hydroxylase Deficiency; Propionic Acidemia; Short Chain Acyl-CoA Dehydrogenase Deficiency-SCADD; Smith-Lemli-Opitz Syndrome; Spinal Muscular Atrophy (SMN1)-SMA; Tay Sachs-HexA Deficiency; Usher Syndrome-Type I (Type IB, Type IC, Type ID, Type IF, Type IG); X-Linked Mental Retardation ARX-Related Disorders; X-Linked Mental Retardation with Cerebellar Cypoplasia and distinctive Facial Appearance; X-Linked Mental Retardation; includes 9, 21, 30, 46, 58, 63, 88, 89; X-linked mental retardation: FM1-Related Disorders-FRXA, Fragile X MR; X-linked SMR: Renpenning Syndrome 1; Zellweger Spectrum disorders-Peroxisomal Bifunctional Enzyme Deficiencies including Zellweger, NALD, and/or infantile Refsums. However, all of these, subsets of these, other genes, or combinations thereof may be used.

According to some aspects, the disclosure relates to multiplex diagnostic methods. In some embodiments, multiplex diagnostic methods comprise capturing a plurality of genetic loci in parallel (e.g., a genetic locus of Table 1). In some embodiments, genetic loci possess one or more polymorphisms (e.g., a polymorphism of Table 2) the genotypes of which correspond to disease causing alleles. Accordingly, in some embodiments, the disclosure provides methods for assessing multiple heritable disorders in parallel.

In some embodiments, methods are provided for diagnosing multiple heritable disorders in parallel at a pre-implantation, prenatal, perinatal, or postnatal stage. In some embodiments, the disclosure provides methods for analyzing multiple genetic loci (e.g., a plurality of target nucleic acids selected from Table 1) from a patient sample, such as a blood, pre-implantation embryo, chorionic villus or amniotic fluid sample. A patient or subject may be a human. However, aspects of the invention are not limited to humans and may be applied to other species (e.g., mammals, birds, reptiles, other vertebrates or invertebrates) as aspects of the invention are not limited in this respect. A subject or patient may be male or female. In some embodiments, in connection with reproductive genetic counseling, samples from a male and female member of a couple may be analyzed. In some embodiments, for example, in connection with an animal breeding program, samples from a plurality of male and female subjects may be analyzed to determine compatible or optimal breeding partners or strategies for particular traits or to avoid one or more diseases or conditions.

However, it should be appreciated that any other diseases may be studied and/or risk factors for diseases or disorders including, but not limited to allergies, responsiveness to treatment, cancer tumor profiling for treatment and prognosis, monitoring and identification of patient infections, and monitoring of environmental pathogens.

1. Reducing Representational Bias in Multiplex Amplification Reactions:

In some embodiments, aspects of the invention relate to methods that reduce bias and increase reproducibility in multiplex detection of genetic loci, e.g., for diagnostic purposes. Molecular inversion probe technology is used to detect or amplify particular nucleic acid sequences in potentially complex mixtures. Use of molecular inversion probes has been demonstrated for detection of single nucleotide polymorphisms (Hardenbol et al. 2005 Genome Res 15:269-75) and for preparative amplification of large sets of exons (Porreca et al. 2007 Nat Methods 4:931-6, Krishnakumar et al. 2008 Proc Natl Acad Sci USA 105:9296-301). One of the main benefits of the method is in its capacity for a high degree of multiplexing, because generally thousands of targets may be captured in a single reaction containing thousands of probes. However, challenges associated with, for example, amplification efficiency (See, e.g., Turner E H, et al., Nat. Methods. 2009 Apr. 6:1-2) have limited the practical utility of the method in research and diagnostic settings.

Aspects of the disclosure are based, in part, on the discovery of effective methods for overcoming challenges associated with systematic errors (bias) in multiplex genomic capture and sequencing methods, namely high variability in target nucleic acid representation and unequal sampling of heterozygous alleles in pools of captured target nucleic acids (e.g., isolated from a biological sample). Accordingly, in some embodiments, the disclosure provides methods that reduce variability in the detection of target nucleic acids in multiplex capture methods. In other embodiments, methods improve allelic representation in a capture pool and, thus, improve variant detection outcomes. In certain embodiments, the disclosure provides preparative methods for capturing target nucleic acids (e.g., genetic loci) that involve the use of different sets of multiple probes (e.g., molecular inversion probes MIPs) that capture overlapping regions of a target nucleic acid to achieve a more uniform representation of the target nucleic acids in a capture pool compared with methods of the prior art. In other embodiments, methods reduce bias, or the risk of bias, associated with large scale parallel capture of genetic loci, e.g., for diagnostic purposes. In other embodiments, methods are provided for increasing reproducibility (e.g., by reducing the effect of polymorphisms on target nucleic acid capture) in the detection of a plurality of genetic loci in parallel. In further embodiments, methods are provided for reducing the effect of probe synthesis and/or probe amplification variability on the analysis of a plurality of genetic loci in parallel.

In some aspects, the disclosure provides probe sets that comprise a plurality of different probes. As used herein, a ‘probe’ is a nucleic acid having a central region flanked by a 5' region and a 3' region that are complementary to nucleic acids flanking the same strand of a target nucleic acid or subregion thereof. An exemplary probe is a molecular inversion probe (MIP). A ‘target nucleic acid’ may be a genetic locus. Exemplary genetic loci are disclosed herein in Table 1 (RefSeqGene Column).

While probes have been typically designed to meet certain constraints (e.g. melting temperature, G/C content, etc.) known to partially affect capture/amplification efficiency (Ball et al (2009) Nat Biotech 27:361-8 AND Deng et al (2009) Nat Biotech 27:353-60), a set of constraints which is sufficient to ensure either largely uniform or highly reproducible capture/amplification efficiency has not previously been achieved. As disclosed herein, uniformity and reproducibility can be increased by designing multiple probes per target, such that each base in the target is captured by more than one probe. In some embodiments, the disclosure provides multiple MIPs per target to be captured, where each MIP in a set designed for a given target nucleic acid has a central region and a 5' region and 3' region (‘targeting arms’) which hybridize to (at least partially) different nucleic acids in the target nucleic acid (immediately flanking a subregion of the target nucleic acid). Thus, differences in efficiency between different targeting arms and fill-in sequences may be averaged across multiple MIPs for a single target, which results in more uniform and reproducible capture efficiency.

In some embodiments, the methods involve designing a single probe for each target (a target can be as small as a single base or as large as a kilobase or more of contiguous sequence).

It may be preferable, in some cases, to design probes to capture molecules (e.g., target nucleic acids or subregions thereof) having lengths in the range of 1-200 bp (as used herein, a by refers to a base pair on a double-stranded nucleic acid-however, where lengths are indicated in bps, it should be appreciated that single-stranded nucleic acids having the same number of bases, as opposed to base pairs, in length also are contemplated by the invention). However, probe design is not so limited. For example, probes can be designed to capture targets having lengths in the range of up to 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 200, 300, 400, 500, 1000, or more bps, in some cases.

It is to be appreciated that the length of a capture molecule (e.g., a target nucleic acid or subregion thereof) is selected based upon multiple considerations. For example, where analysis of a target involves sequencing, e.g., with a next-generation sequencer, the target length should typically match the sequencing read-length so that shotgun library construction is not necessary. However, it should be appreciated that captured nucleic acids may be sequenced using any suitable sequencing technique as aspects of the invention are not limited in this respect.

It is also to be appreciated that some target nucleic acids are too large to be captured with one probe. Consequently, it may be necessary to capture multiple subregions of a target nucleic acid in order to analyze the full target.

In some embodiments, a subregion of a target nucleic acid is at least 1 bp. In other embodiments, a subregion of a target nucleic acid is at least 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000 bp or more. In other embodiments, a subregion of a target nucleic acid has a length that is up to 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, or more percent of a target nucleic acid length.

The skilled artisan will also appreciate that consideration is made, in the design of MIPs, for the relationship between probe length and target length. In some embodiments, MIPs are designed such that they are several hundred basepairs (e.g., up to 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000 bp or more) longer than corresponding target (e.g., subregion of a target nucleic acid, target nucleic acid).

In some embodiments, lengths of subregions of a target nucleic acid may differ. For example, if a target nucleic acid contains regions for which probe hybridization is not possible or inefficient, it may be necessary to use probes that capture subregions of one or more different lengths in order to avoid hybridization with problematic nucleic acids and capture nucleic acids that encompass a complete target nucleic acid.

Aspects of the invention involve using multiple probes, e.g., MIPs, to amplify each target nucleic acid. In some embodiments, the set of probes for a given target can be designed to 'tile' across the target, capturing the target as a series of shorter sub-targets. In some embodiments, where a set of probes for a given target is designed to 'tile' across the target, some probes in the set capture flanking non-target sequence). Alternately, the set can be designed to 'stagger' the exact positions of the hybridization regions flanking the target, capturing the full target (and in some cases capturing flanking non-target sequence) with multiple probes having different targeting arms, obviating the need for tiling. The particular approach chosen will depend on the nature of the target set. For example, if small regions are to be captured, a staggered-end approach might be appropriate, whereas if longer regions are desired, tiling might be chosen. In all cases, the amount of bias-tolerance for probes targeting pathological loci can be adjusted ('dialed in') by changing the number of different MIPs used to capture a given molecule. In some embodiments, the 'coverage factor', or number of probes used to capture a basepair in a molecule, is an important parameter to specify. Different numbers of probes per target are indicated depending on whether one is using the tiling approach (see, e.g., FIG. **1**) or one of the staggered approaches (see, e.g., FIG. **2** or **3**).

FIG. **1** illustrates a non-limiting embodiment of a tiled probe layout showing ten captured sub-targets tiled across a single target. Each position in the target is covered by three sub-targets such that MIP performance per base pair is averaged across three probes.

FIG. **2** illustrates a non-limiting embodiment of a staggered probe layout showing the targets captured by a set of three MIPs. Each MIP captures the full target, shown in black, plus (in some cases) additional extra-target sequence, shown in gray, such that the targeting arms of each MIP fall on different sequence. Each position in the target is covered by three sub-targets such that MIP performance per basepair is averaged across three probes. Targeting arms land immediately adjacent to the black or gray regions shown. It should be appreciated that in some embodiments, the targeting arms (not shown) can be designed so that they do not overlap with each other.

FIG. **3** illustrates a non-limiting embodiment of an alternating staggered probe layout showing the targets captured by a set of three MIPs. Each MIP captures the full target, shown in black, plus (in some cases) additional extra-target sequence, shown in gray, such that the targeting arms of each MIP fall on different sequence. Each position in the target is covered by three sub-targets such that MIP performance per basepair is averaged across three probes. Targeting arms land immediately adjacent to the black or gray regions shown.

It should be appreciated that for any of the layouts, the targeting arms on adjacent tiled or staggered probes may be designed to either overlap, not overlap, or overlap for only a subset of the probes.

In certain embodiments for any of the layouts, a coverage factor of about 3 to to about 10 is used. However, the methods are not so limited and coverage factors of up to 2, 3, 4, 5, 6, 7, 8, 9, 10, 20 or more may be used. It is to be appreciated that the coverage factor selected may depend the probe layout being employed. For example, in the tiling approach, for a desired coverage factor, the number of probes per target is typically a function of target length, sub-target length, and spacing between adjacent sub-target start locations (step size). For example, for a desired coverage factor of 3, a 200 bp target with a start-site separation of 20 bp and sub-target length of 60 bp may be encompassed with 12 MIPs (FIG. **1**). Thus, a specific coverage factor may be achieved by varying the number of probes per target nucleic acid and the length of the molecules captured. In the staggered approach, a fixed-length target nucleic acid is captured as several subregions or as 'super-targets', which are molecules comprising the target nucleic acid and additional flanking nucleic acids, which may be of varying lengths. For example, a target of 50 bp can be captured at a coverage factor of 3 with 3 probes in either a 'staggered' (FIG. **2**) or 'alternating staggered' configuration (FIG. **3**).

The coverage factor will be driven by the extent to which detection bias is tolerable. In some cases, where the bias tolerance is small, it may be desirable to target more subregions of target nucleic acid with, perhaps, higher coverage factors. In some embodiments, the coverage factor is up to 2, 3, 4, 5, 6, 7, 8, 9, 10 or more.

In some embodiments, when a tiled probe layout is used, when the target length is greater than 1 bp and when a step size (distance between the 5'-end of a target and the 5' end of its adjacent target) is less than the length of a target or subregion thereof, it is possible to compute probe number for a particular target based on target length (T), sub-target length (S), and coverage factor (C), such that probe number=T/(S/C)+(C−1).

In some aspects, the disclosure provides methods to increase the uniformity of amplification efficiency when multiple molecules are amplified in parallel; methods to increase the reproducibility of amplification efficiency; methods to reduce the contribution of targeting probe variability to amplification efficiency; methods to reduce the effect on a given target nucleic acid of polymorphisms in probe hybridization regions; and/or methods to simplify downstream workflows when multiplex amplification by MIPs is used as a preparative step for analysis by nucleic acid sequencing.

Polymorphisms in the target nucleic acid under the regions flanking a target can interfere with hybridization, polymerase fill-in, and/or ligation. Furthermore, this may occur for only one allele, resulting in allelic drop-out, which ultimately decreases downstream sequencing accuracy. In some embodiments, using a set of MIPs having multiple hybridization sites for the capture of any given target, the probability of loss from polymorphism is substantially decreased because not all targeting arms in the set of MIPs will cover the location of the mutation.

Probes for MIP capture reactions may be synthesized on programmable microarrays because of the large number of sequences required. Because of the low synthesis yields of these methods, a subsequent amplification step is required to produce sufficient probe for the MIP amplification reaction. The combination of multiplex oligonucleotide synthesis and pooled amplification results in uneven synthesis error rates and representational biases. By synthesizing multiple probes for each target, variation from these sources may be averaged out because not all probes for a given target will have the same error rates and biases.

Multiplex amplification strategies disclosed herein may be used analytically, as in detection of SNPs, or preparatively, often for next-generation sequencing or other sequencing techniques. In the preparative setting, the output of an amplification reaction is generally the input to a shotgun library protocol, which then becomes the input to the sequencing platform. The shotgun library is necessary in part because next-generation sequencing yields reads significantly shorter than amplicons such as exons. In addition to the bias-reduction afforded by the multi-tiled approach described here, tiling also obviates the need for shotgun library preparation. Since the length of the capture molecule can be specified when the probes, e.g., MIPs, are designed, it can be chosen to match the readlength of the sequencer. In this way, reads can 'walk' across an exon by virtue of the start position of each capture molecule in the probe set for that exon.

Exemplary molecular inversion probes are provided in Appendix A. These molecular inversion probes are designed to capture targets or sub-regions thereof on one or more genes listed in Table 5 (provided in Example 8). In certain applications, the molecular inversion probes provided in Appendix A may be used to tile-capture targets or sub-regions thereof on the one or more genes provided in Table 5. In particular applications, two or more of the molecular inversion probes of Appendix A tile across different, but overlapping sub-regions of one or more genes listed in Table 5 so that a target on the gene is capture by both of the two or more molecular inversion probes, as exemplified in FIG. **1**.

In certain embodiments, the molecular inversion probes of Appendix A that are chosen for tile-capture a target depends on the desired amount of overlapping coverage for the target. In one example, two or more molecular inversion probes of Appendix A, being in directly ascending SEQ ID NO: order and corresponding to a target nucleic acid, will tile across the target nucleic acid with a period of 25 base pairs such that every genomic position of the target nucleic acid is capture by multiple probes with orthogonal targeting arm sequences. If less coverage is desired for a target nucleic acid, one may select, for example, every other molecular inversion probes of Appendix A in ascending order that correspond to that target.

The first and second targeting arms of the molecular inversion probes are designed to hybridize to nucleotides upstream and downstream of a capture region of a gene (i.e. the targeting arms flank the region to be captured). The capture region may be a target nucleic acid or a sub-region thereof. Appendix B lists the capture regions of the genes that correspond to the molecular inversion probes listed in Appendix A. Appendix A also specifies the upstream and downstream regions of the capture regions corresponding to each targeting arm of the molecular inversion probes. The upstream and downstream regions of the capture region are between the start position and the end position coordinates, which are relative to the Human Genome 18 (HG 18).

The molecular inversion probes of Appendix A include a central region flanked by a 5' first targeting arm (i.e. ligation arm or left arm) and a 3' second targeting arm (i.e. extension arm or right arm). The targeting arm sequences are shown in lowercase letters and the central region sequence is shown in uppercase letters. The 5' first targeting arm and the 3' second targeting arm of the molecular inversion probes provided in Appendix A include a total of 40 nucleotides, and are designed to flank 130 bp capture regions. Some of the molecular inversion probes listed in Appendix A are designed to capture the coding regions of the genes, whereas others are designed to capture non-coding regions of the genes. The genes listed in Table 5 corresponded to diseases, and as such, the molecular inversion probes listed in Appendix A can be utilized to analyze one or more of the diseases provided in table 5. The molecular inversion probes provided in Appendix A are described in more detail in Example 8.

While all of the molecular inversion probes provided in Appendix A may be used in a single assay to comprehensively examine several or all of the genomic regions of the genes provided in Table 5, one may also select one or more molecular inversion probes provided in Appendix A to evaluate one or more targets present in one gene or a combination of the genes provided in Table 5. For example, one may choose to only examine the coding regions of one or more of the genes listed in Table 5, and therefore use the one or more of the molecular inversion probes designed to capture those regions. In another example, one may choose to only examine the non-coding regions of one or more gene listed in Table 5, and therefore use the one or more molecular inversion probes designed to capture those regions. In another example, one may choose to only examine a portion of or the entirety of a gene listed in Table 5, and therefore use the one or more molecular inversion probes design to capture the portion of or the entirety of that gene. In another example, one may choose to examine nucleic acid regions specific to one or more diseases listed in Table 5, and therefore use the one or more nucleic acids corresponding to those diseases. In yet another example, one may choose to examine a portion or entirety of two or more of the genes listed in Table 5, and there uses the molecular inversion probes specific to those genes. In yet another example, one may choose to only examine certain chromosomes with the molecular inversion probes provided in Appendix A. In all of these examples, the number of molecular inversion probes that correspond to the target chosen depends on the amount of coverage one desires.

It is understood that one can modify the molecular inversion probes listed in Appendix A, while achieving a similar coverage and tile-capture layout as the probes listed in Appendix A. For example, the sequence of the central region of the molecular inversion probes may be different from the sequence of the central region provided in Appendix A without changing capture region of the probe. For molecular inversion probe sets, the sequence chosen for the central region is preferably the same across each molecular inversion probe in a set of probes. This allows the capture targets to be amplified with a single set of primers. It is also preferable that the central region is designed so that it is not complementary to the target sequences or any other sequence in the sample.

In addition, it should be appreciated that other molecular inversion probes than those listed in Appendix A may be used to tile-capture different regions of the genes listed in Table 5. Those molecular inversion probes may include a different first targeting arm, second targeting arm, and/or central region from the molecular inversion probes listed in Appendix A. In a non-limiting example, a modified molecular inversion probe may include the first targeting arm sequence of SEQ ID NO: 300, but have a different sequence for the central region and the second targeting arm. The specific sequences and length of the sequences chosen for the first targeting arm, second targeting arm, and/or central region depend on the desired capture region and coverage.

In certain embodiments, the molecular inversion probes for tile or staggered capture are selected to maximize performance with respect to both capture efficiency and robustness to common polymorphisms. In order to determine which probes maximize performance for a genomic target, methods of the invention, according to certain aspects, involve designing all possible probes capable of targeting a genomic interval and ranking the probes based on a number of score tuples or ranking factors. In certain embodiments, the possible probes are assigned score tuples including, but not limited to: 1) presence of guanine or cytosine as the 5'-most base of the ligation arm, 2) the number of dbSNP (version 130) entries intersecting targeting arm sites, 3) the root mean squared deviation of the targeting arms' predicted melting temperatures from optimal values derived from empirical studies of efficiencies. Using any combination of these score tuples, the possible probes for a certain genomic interval may be ranked, and the highest ranking probe for the genomic interval is preferably chosen for capture.

In certain aspects, method of the invention provide for shearing or fragmenting genomic nucleic acid prior to performing capture with a molecular inversion probe (e.g. capture with one or more of the molecular inversion probes provided in Appendix A). Fragmenting the genomic nucleic acid prior to performing a capture reaction allows for greater exposure of a target site to a molecular inversion probe, which reduces failed capture and increases the percentage of molecular inversion probes that hybridize to targets within the genome. This advantageously yields a target abundance distribution that is significantly more uniform than if a native high molecular weight genomic nucleic acid is used. Molecular inversion techniques involving a fragmenting step are described in co-owned and co-assigned U.S. Ser. No. 13/448,961, having U.S. Publication No. 2012/0252020, entitled "Capture Reactions."

Fragmenting the nucleic acid can be accomplished by any technique known in the art. Exemplary techniques include mechanically fragmenting, chemically fragmenting, and/or enzymatically fragmenting. Mechanical nucleic acid fragmentation can be, for example, sonication, nebulization, and hydro-shearing (e.g., point-sink shearing). Enzymatic nucleic acid fragmenting includes, for example, use of nicking endonucleases or restriction endonucleases. The nucleic acid can also be chemically fragmented by performing acid hydrolysis on the nucleic acid or treating of the nucleic acid with alkali or other reagents.

The fragment length can be adjusted based on the sizes of the nucleic acid targets to be captured. The nucleic acid fragments can be of uniform length or of a distribution of lengths. In certain embodiments, the nucleic acid is fragmented into nucleic acid fragments having a length of about 10 kb or 20 kb. In addition, the nucleic acid fragments can range from between 1 kb to 20 kb, with various distributions.

In certain embodiments, the nucleic acid is also denatured, which may occur prior to, during, or after the fragmenting step. The nucleic acid can be denatured using any means known in the art, such as pH-based denaturing, heat-based denaturing, formamide or urea, exonuclease degradation, or endonuclease nicking. In certain embodiments, the use of pH, such as in acid hydrolysis, alone or in combination with heat fragments and either partially or fully denatures the nucleic acid. This combined fragmenting and denaturing method can be used to fragment the nucleic acid for MIP capture or to fragment captured target nucleic acids or whole genomic DNA for shotgun library preparation.

In one aspect, a nucleic acid is fragmented by heating a nucleic acid immersed in a buffer system at a certain temperature for a certain period to time to initiate hydrolysis and thus fragment the nucleic acid. The pH of the buffer system, duration of heating, and temperature can be varied to achieve a desired fragmentation of the nucleic acid. In one embodiment, after a genomic nucleic acid is purified, it is resuspended in a Tris-based buffer at a pH between 7.5 and 8.0, such as Qiagen's DNA hydrating solution. The resuspended genomic nucleic acid is then heated to 65° C. and incubated overnight (about 16-24 hours) at 65° C. Heating shifts the pH of the buffer into the low- to mid-6 range, which leads to acid hydrolysis. Over time, the acid hydrolysis causes the genomic nucleic acid to fragment into single-stranded and/or double-stranded products. The above method of fragmenting can be modified by increasing the temperature and reducing the heating time. For example, a nucleic acid is fragmented by incubating the nucleic acid in the Tris-based buffer at a pH between 7.5 and 8.0 for 15 minutes at 92° C. In addition to adjusting the temperature and the duration of heating, the pH of the Tris-based buffer can be adjusted to achieve a desired nucleic acid fragmentation.

Once molecular inversion probes of the invention are hybridized to genomic or fragmented nucleic acid, the captured target may further be subjected to an enzymatic gap-filling and ligation step, such that a copy of the target sequence is incorporated into a circle. Capture efficiency of the MIP to the target sequence on the nucleic acid fragment can be improved by lengthening the hybridization and gap-filing incubation periods. (See, e.g., Turner E H, et al., Nat Methods. 2009 Apr. 6:1-2).

The result of molecular inversion probe capture as described above is a library of circular target probes, which then can be processed in a variety of ways. In one aspect, adaptors for sequencing can be attached during common linker-mediated PCR, resulting in a library with non-random, fixed starting points for sequencing. In another aspect, for preparation of a shotgun library, a common linker-mediated PCR is performed on the circle target probes, and the post-capture amplicons are linearly concatenated, sheared, and attached to adaptors for sequencing. Methods for shearing the linear concatenated captured targets can include any of the methods disclosed for fragmenting nucleic acids discussed above. In certain aspects, performing a hydrolysis reaction on the captured amplicons in the presence of heat is the desired method of shearing for library production.

Sequencing may be by any method known in the art. DNA sequencing techniques include classic dideoxy sequencing reactions (Sanger method) using labeled terminators or primers and gel separation in slab or capillary, sequencing by synthesis using reversibly terminated labeled nucleotides, pyrosequencing, 454 sequencing, Illumina/Solexa sequencing, allele specific hybridization to a library of labeled oligonucleotide probes, sequencing by synthesis using allele specific hybridization to a library of labeled clones that is followed by ligation, real time monitoring of the incorporation of labeled nucleotides during a polymerization step, polony sequencing, and SOLiD sequencing. Separated molecules may be sequenced by sequential or single extension reactions using polymerases or ligases as well as by single or sequential differential hybridizations with libraries of probes.

An example of a sequencing technology that can be used is Illumina sequencing. Illumina sequencing is based on the amplification of DNA on a solid surface using fold-back PCR and anchored primers. Genomic DNA is fragmented, and adapters are added to the 5' and 3' ends of the fragments. DNA fragments that are attached to the surface of flow cell channels are extended and bridge amplified. The fragments become double stranded, and the double stranded molecules are denatured. Multiple cycles of the solid-phase amplification followed by denaturation can create several million clusters of approximately 1,000 copies of single-stranded DNA molecules of the same template in each channel of the flow cell. Primers, DNA polymerase and four fluorophore-labeled, reversibly terminating nucleotides are used to perform sequential sequencing. After nucleotide incorporation, a laser is used to excite the fluorophores, and an image is captured and the identity of the first base is recorded. The 3' terminators and fluorophores from each incorporated base are removed and the incorporation, detection and identification steps are repeated. Sequencing according to this technology is described in U.S. Pat. Nos. 7,960,120; 7,835,871; 7,232,656; 7,598,035; 6,911,345; 6,833,246; 6,828,100; 6,306,597; 6,210,891; U.S. Pub. 2011/0009278; U.S. Pub. 2007/0114362; U.S. Pub. 2006/0292611; and U.S. Pub. 2006/0024681, each of which are incorporated by reference in their entirety.

Sequencing generates a plurality of reads. Reads generally include sequences of nucleotide data less than about 150 bases in length, or less than about 90 bases in length. In certain embodiments, reads are between about 80 and about 90 bases, e.g., about 85 bases in length. In some embodiments, these are very short reads, i.e., less than about 50 or about 30 bases in length. A set of sequence reads can be analyzed by any suitable method known in the art. For example, in some embodiments, sequence reads are analyzed by hardware or software provided as part of a sequence instrument. In some embodiments, individual sequence reads are reviewed by sight (e.g., on a computer monitor). A computer program may be written that pulls an observed genotype from individual reads. In certain embodiments, analyzing the reads includes assembling the sequence reads and then genotyping the assembled reads.

In certain embodiments, the sequences obtained using the molecular inversion probe techniques of the invention are analyzed using the methods for evaluating of genetic test, which are described in U.S. Provisional Ser. No. 61/723,508, entitled “Validation of Genetic Test.” The method involves obtaining a plurality of sequence reads, introducing a simulated mutation into at least one of the plurality of sequence reads, and analyzing the sequence reads to determine if the test identifies the simulated mutation. To mimic the expected genotype of a heterozygous carrier, the simulated mutation can be introduced into each of those sequence reads that span a location of the mutation with a probability of 0.5 (e.g., into about half of those sequence reads that should contain the location of the simulated mutation). The simulated mutation can be introduced by manipulating a data field in the sequence read such as, for example, a base sequence field or quality data field. The sequences can be manipulated by a computer program. For example, a program can be written using Java, Groovy, Python, Perl, or other languages, or a combination thereof, that can automatically insert simulated mutations into sequence reads. Computer-based methods can be used to automatically introduce a number of different simulated mutations into different ones of the plurality of sequence reads.

The sequence reads including the manipulated reads are analyzed to detect a genotype. Analysis can include any method known in the art, such as de nova assembly, alignment to a reference, or a combination thereof. In some embodiments, the sequence reads are assembled into a contig. The contig can be aligned to a reference genome. In certain embodiments, individual reads are then aligned back to the contig.

Sequence assembly can be done by methods known in the art including reference-based assemblies, de novo assemblies, assembly by alignment, or combination methods. Assembly can include methods described in U.S. Pat. No. 8,209,130 titled Sequence Assembly by Porecca and Kennedy, the contents of each of which are hereby incorporated by reference in their entirety for all purposes. In some embodiments, sequence assembly uses the low coverage sequence assembly software (LOCAS) tool described by Klein, et al., in LOCAS-A low coverage sequence assembly tool for re-sequencing projects, PLoS One 6(8) article 23455 (2011), the contents of which are hereby incorporated by reference in their entirety. Sequence assembly is described in U.S. Pat. Nos. 8,165,821; 7,809,509; 6,223,128; U.S. Pub. 2011/0257889; and U.S. Pub. 2009/0318310, the contents of each of which are hereby incorporated by reference in their entirety.

In certain embodiments, genetic test of the invention are validated using a genotyping by assembly-template alignment (GATA) technique, which is also described in co-pending and co-owned U.S. Provisional Ser. No. 61/723,508, entitled “Validation of Genetic Test.” FIG. **21** diagrams the validation of a genotyping by assembly-templated alignment (GATA) technique. Genetic analysis by GATA-based methods includes obtaining 401 sequence reads and assembling **405** the reads into a contig, which is then aligned 409 to a reference. Differences are identified by comparison 413. The raw reads are aligned 417 to the contigs and positional and variant information is mapped to the reads from the reference via the contig, allowing genotyping 421 to produce an observed genotyping. The GATA-based method is evaluated by introducing 403 at least one simulated mutation into the reads.

FIG. **22** illustrates obtaining sequence reads and inserting a simulated mutation. As shown in FIG. **22**, if only wild type sample is sequenced, the raw sequence reads may only include wild type sequence. However, a mutation of interest may be known, for example, from the literature or it may be desirable to simply invent a difficult-to-detect mutation to use in methods of validating a genetic analysis. Here, a hypothetical 8 base pair deletion proximal to a C>A substitution is depicted. As shown in FIG. **22**, the raw sequence reads are edited so that they include base sequence data, quality data, or both that would arise from sequencing the simulated mutation.

FIG. **23** shows an example in which a standard analytical method is performed for comparison to a GATA-based method. The standard analysis is demonstrated to not be able to detect a mutation. FIG. **23** depicts a workflow in which edited sequence reads (e.g., as depicted in FIG. **22**) are aligned to a reference genome (here, using BWA and GATK). The alignment software properly aligns the wild type sequence reads to the reference genome, finding a perfect match and giving a result indicating that the sample is the wild type. However, the alignment software finds no valid alignment for the edited sequence reads and is unable to produce a result. Due to the fact that the expected genotype of the edited sequence reads is known a priori (and, in fact intentionally supplied by editing), an operator is able to identify that this analysis method-alignment of sequence reads to a reference genome—is incapable of detecting the mutation. For comparison, the sequence reads are also analyzed by a GATA-based method.

FIG. **24** shows analysis of sequence reads that include simulated mutations by GATA. In step 1, reads are assembled into contigs. Assembly can include any method including those discussed below. In step 2, each contig is aligned to a reference genome. Alignment can be by any method such as those discussed below, including, e.g., the bwa-sw algorithm implemented by BWA. As shown in FIG. **24**, both align to the same reference position. Differences between the contig and the reference genome are identified and, as shown in FIG. **26**, described by a CIGAR string.

In step 3, raw reads are aligned to contigs (using any method such as, for example, BWA with bwa-short and writing, for example, a CIGAR string). At step 4, raw read alignments are mapped from contig space to original reference space (e.g., via position and CIGAR information). In step 5, genotyping is performed using the translated, aligned reads from step 4 (e.g., including raw quality scores for substitutions).

For step 1, reads may be assembled into contigs by any method known in the art. Algorithms for the de nova assembly of a plurality of sequence reads are known in the art. One algorithm for assembling sequence reads is known as overlap consensus assembly. Assembly with overlap graphs is described, for example, in U.S. Pat. No. 6,714,874. In some embodiments, de nova assembly proceeds according to so-called greedy algorithms, as described in U.S. Pub. 2011/0257889, incorporated by reference in its entirety. In other embodiments, assembly proceeds by either exhaustive or heuristic pairwise alignment. Exhaustive pairwise alignment, sometimes called a "brute force" approach, calculates an alignment score for every possible alignment between every possible pair of sequences among a set. Assembly by heuristic multiple sequence alignment ignores certain mathematically unlikely combinations and can be computationally faster. One heuristic method of assembly by multiple sequence alignment is the so-called "divide-and-conquer" heuristic, which is described, for example, in U.S. Pub. 2003/0224384. Another heuristic method of assembly by multiple sequence alignment is progressive alignment, as implemented by the program ClustalW (see, e.g., Thompson, et al., Nucl. Acids. Res., 22:4673-80 (1994)).

With continuing reference to step 1 of FIG. **24**, in some embodiments assembly into contigs involves making a de Bruijn graph. De Bruijn graphs reduce the computation effort by breaking reads into smaller sequences of DNA, called k-mers, where the parameter k denotes the length in bases of these sequences. In a de Bruijn graph, all reads are broken into k-mers (all subsequences of length k within the reads) and a path between the k-mers is calculated. In assembly according to this method, the reads are represented as a path through the k-mers. The de Bruijn graph captures overlaps of length k−1 between these k-mers and not between the actual reads. By reducing the entire data set down to k-mer overlaps, the de Bruijn graph reduces the high redundancy in short-read data sets. Assembly of reads using de Bruijn graphs is described in U.S. Pub. 2011/0004413, U.S. Pub. 2011/0015863, and U.S. Pub. 2010/0063742, incorporated by reference in their entirety. Assembly of reads into contigs is further discussed in U.S. Pat. No. 6,223,128, U.S. Pub. 2009/0298064, U.S. Pub. 2010/0069263, and U.S. Pub. 2011/0257889, each of which is incorporated by reference herein in its entirety.

2. Reducing Analytical Errors Associated with Bias in Nucleic Acid Preparations:

In some embodiments, aspects of the invention relate to preparative steps in DNA sequencing-related technologies that reduce bias and increase the reliability and accuracy of downstream quantitative applications.

There are currently many genomics assays that utilize next-generation (polony-based) sequencing to generate data, including genome resequencing, RNA-seq for gene expression, bisulphite sequencing for methylation, and Immune-seq, among others. In order to make quantitative measurements (including genotype calling), these methods utilize the counts of sequencing reads of a given genomic locus as a proxy for the representation of that sequence in the original sample of nucleic acids. The majority of these techniques require a preparative step to construct a high-complexity library of DNA molecules that is representative of a sample of interest. This may include chemical or biochemical treatment of the DNA (e.g., bisulphite treatment), capture of a specific subset of the genome (e.g., padlock probe capture, solution hybridization), and a variety of amplification techniques (e.g., polymerase chain reaction, whole genome amplification, rolling circle amplification).

Systematic and random errors are common problems associated with genome amplification and sequencing library construction techniques. For example, genomic sequencing library may contain an over- or under-representation of particular sequences from a source genome as a result of errors (bias) in the library construction process. Such bias can be particularly problematic when it results in target sequences from a genome being absent or undetectable in the sequencing libraries. For example, an under-representation of particular allelic sequences (e.g., heterozygotic alleles) from a genome in a sequencing library can result in an apparent homozygous representation in a sequencing library. As most downstream sequencing library quantification techniques depend on stochastic counting processes, these problems have typically been addressed by sampling enough (over-sampling) to obtain a minimum number of observations necessary to make statistically significant decisions. However, the strategy of oversampling is generally limited to elimination of low-count Poisson noise, and the approach wastes resources and increases the expense required to perform such experiments. Moreover, oversampling can result in a reduced statistical confidence in certain conclusions (e.g., diagnostic calls) based on the data. Accordingly, new approaches are needed for overcoming bias in sequencing library preparatory methods.

Aspects of the disclosure are based, in part, on the discovery of methods for overcoming problems associated with systematic and random errors (bias) in genome capture, amplification and sequencing methods, namely high variability in the capture and amplification of nucleic acids and disproportionate representation of heterozygous alleles in sequencing libraries. Accordingly, in some embodiments, the disclosure provides methods that reduce variability in the capture and amplification of nucleic acids. In other embodiments, the methods improve allelic representation in sequencing libraries and, thus, improve variant detection outcomes. In certain embodiments, the disclosure provides preparative methods for capturing target nucleic acids (e.g., genetic loci) that involve the use of differentiator tag sequences to uniquely tag individual nucleic acid molecules. In some embodiments, the differentiator tag sequence permits the detection of bias based on the frequency with which pairs of differentiator tag and target sequences are observed in a sequencing reaction. In other embodiments, the methods reduce errors caused by bias, or the risk of bias, associated with the capture, amplification and sequencing of genetic loci, e.g., for diagnostic purposes.

Aspects of the invention relate to associating unique sequence tags (referred to as differentiator tag sequences) with individual target molecules that are independently captured and/or analyzed (e.g., prior to amplification or other process that may introduce bias). These tags are useful to distinguish independent target molecules from each other thereby allowing an analysis to be based on a known number of individual target molecules. For example, if each of a plurality of target molecule sequences obtained in an assay is associated with a different differentiator tag, then the target sequences can be considered to be independent of each other and a genotype likelihood can be determined based on this information. In contrast, if each of the plurality of target molecule sequences obtained in the assay is associated with the same differentiator tag, then they probably all originated from the same target molecule due to over-representation (e.g., due to biased amplification) of this target molecule in the assay. This provides less information than the situation where each nucleic acid was associated with a different differentiator tag. In some embodiments, a threshold number of independently isolated molecules (e.g., unique combinations of differentiator tag and target sequences) is analyzed to determine the genotype of a subject.

In some embodiments, the invention relates to compositions comprising pools (libraries) of preparative nucleic acids that each comprise "differentiator tag sequences" for detecting and reducing the effects of bias, and for genotyping target nucleic acid sequences. As used herein, a "differentiator tag sequence" is a sequence of a nucleic acid (a preparative nucleic acid), which in the context of a plurality of different isolated nucleic acids, identifies a unique, independently isolated nucleic acid. Typically, differentiator tag sequences are used to identify the origin of a target nucleic acid at one or more stages of a nucleic acid preparative method. For example, in the context of a multiplex nucleic acid capture reaction, differentiator tag sequences provide a basis for differentiating between multiple independent, target nucleic acid capture events. Also, in the context of a multiplex nucleic acid amplification reaction, differentiator tag sequences provide a basis for differentiating between multiple independent, primary amplicons of a target nucleic acid, for example. Thus, combinations of target nucleic acid and differentiator tag sequence (target:differentiator tag sequences) of an isolated nucleic acid of a preparative method provide a basis for identifying unique, independently isolated target nucleic acids. FIG. **4**A-C depict various non-limiting examples of methods for combining differentiator tag sequence and target sequences.

It will be apparent to the skilled artisan that differentiator tags may be synthesized using any one of a number of different methods known in the art. For example, differentiator tags may be synthesized by random nucleotide addition. Differentiator tag sequences are typically of a predefined length, which is selected to control the likelihood of producing unique target:differentiator tag sequences in a preparative reaction (e.g., amplification-based reaction, a circularization selection-based reaction, e.g., a MIP reaction). Differentiator tag sequences may be, up to 5, up to 6, up to 7 up to 8, up to 9, up to 10, up to 11, up to 12, up to 13, up to 14, up to 15, up to 16, up to 17, up to 18, up to 19, up to 20, up to 21, up to 22, up to 23, up to 24, up to 25, or more nucleotides in length. For purposes of genotyping, isolated nucleic acids are identified as independently isolated if they comprise unique combinations of target nucleic acid and differentiator tag sequences, and observance of threshold numbers of unique combinations of target nucleic acid and differentiator tag sequences provide a certain statistical confidence in the genotype.

During a library preparation process, each nucleic acid molecule may be tagged with a unique differentiator tag sequence in a configuration that permits the differentiator tag sequence to be sequenced along with the target nucleic acid sequence of interest (the nucleic acid sequence for which the library is being prepared, e.g., a polymorphic sequence). The incorporation of the nucleic acid comprising a differentiator tag sequence at a particular step allows the detection and correction of biases in subsequent steps of the protocol.

A large library of unique differentiator tag sequences may be created by using degenerate, random-sequence polynucleotides of defined length. The differentiator tag sequences of the polynucleotides may be read at the final stage of the sequencing. The observations of the differentiator tag sequences may be used to detect and correct biases in the final sequencing read-out of the library. For example, the total possible number of differentiator tag sequences, which may be produced, e.g., randomly, is 4N, where N is the length of the differentiator tag sequence. Thus, it is to be understood that the length of the differentiator tag sequence may be adjusted such that the size of the population of MIPs having unique differentiator tag sequences is sufficient to produce a library of MIP capture products in which identical independent combinations of target nucleic acid and differentiator tag sequence are rare. As used herein combinations of target nucleic acid and differentiator tag sequences, may also be referred to as "target:differentiator tag sequences".

In the final readout of a sequencing process, each read may have an additional unique differentiator tag sequence. In some embodiments, when differentiator tag sequences are distributed randomly in a library, all the unique differentiator tag sequences will be observed about an equal number of times. Accordingly, the number of occurrences of a differentiator tag sequence may follow a Poisson distribution.

In some embodiments, overrepresentation of target:differentiator tag sequences in a pool of preparative nucleic acids (e.g., amplified MIP capture products) is indicative of bias in the preparative process (e.g., bias in the amplification process). For example, target:differentiator tag sequence combinations that are statistically overrepresented are indicative of bias in the protocol at one or more steps between the incorporation of the differentiator tag sequences into MIPs and the actual sequencing of the MIP capture products.

The number of reads of a given target:differentiator tag sequence may be indicative (may serve as a proxy) of the amount of that target sequence present in the originating sample. In some embodiments, the numbers of occurrence of sequences in the originating sample is the quantity of interest. For example, using the methods disclosed herein, the occurrence of differentiator tag sequences in a pool of MIPs may be predetermined (e.g., may be the same for all differentiator tag sequences). Accordingly, changes in the occurrence of differentiator tag sequences after amplification and sequencing may be indicative of bias in the protocol. Bias may be corrected to provide an accurate representation of the composition of the original MIP pool, e.g., for diagnostic purposes.

According to some aspects, a library of preparative nucleic acid molecules (e.g., MIPs, each nucleic acid in the library having a unique differentiator tag sequence, may be constructed such that the number of nucleic acid molecules in the library is significantly larger than the number prospective target nucleic acid molecules to be captured using the library. This ensures that products of the preparative methods include only unique target:differentiator tag sequence; e.g., in a MIP reaction the capture step would undersample the total population of unique differentiator tag sequences in the MIP library. For example, an experiment utilizing 1 μg of genomic DNA will contain about ~150,000 copies of a diploid genome. For a MIP library, each MIP in the library comprising a randomly produced 12-mer differentiator tag sequence (~4.6 million possible unique differentiator tag sequences), there would be more than 100 unique differentiator tag sequences per genomic copy. For a MIP library, each MIP in the library comprising a randomly produced 15-mer differentiator tag sequence C1 billion possible unique differentiator tag sequences), there would be more than 7000 unique differentiator tag sequences per genomic copy. Therefore, the probability of the same differentiator tag sequence being incorporated multiple times is incredibly small. Thus, it is to be appreciated that the length of the differentiator tag sequence is to be selected based on the amount of target sequence in a MIP capture reaction and the desired probability for having multiple, independent occurrences of target:differentiator tag sequence combinations.

FIG. **5** depicts a non-limiting method for genotyping based on target and differentiator tag sequences. Sequencing reads of target and differentiator tags sequences are collapsed to make diploid genotype calls. FIG. **6** depicts non-limiting results of a simulation of a MIP capture reaction in which MIP probes, each having a differentiator tag sequence of 15 nucleotides, are combined with 10000 target sequence copies (e.g., genome equivalents). In this simulated reaction, the probability of capturing one or more copies of a target sequence having the same differentiator tag sequence is 0.05. The Y axis reflects the number of observations. The X axis reflects the number of independent occurrences of target:differentiator tag combinations. FIG. **7** depicts a non-limiting graph of sequencing coverage, which can help ensure that alleles are sampled to sufficient depth (e.g., either 10× or 20× minimum sampling per allele, assuming 1000 targets). In this non-limiting example, the X axis is total per-target coverage required, and the Y axis is the probability that a given total coverage will result in at least 10× or 20× coverage for each allele.

The skilled artisan will appreciate that as part of a MIP library preparation process, adapters may be ligated onto the ends of the molecules of interest. Adapters often contain PCR primer sites (for amplification or emulsion PCR) and/or sequencing primer sites. In addition, barcodes may be included, for example, to uniquely identify individual samples (e.g., patient samples) that may be mixed together. (See, e.g., USPTO Publication Number US 2007/0020640 A1 (McCloskey et al) The actual incorporation of the random differentiator tag sequences can be performed through various methods known in the art. For example, nucleic acids comprising differentiator tag sequences may be incorporated by ligation. This is a flexible method, because molecules having differentiator tag sequence can be ligated to any blunt-ended nucleic acids. The sequencing primers must be incorporated subsequently such that they sequence both the differentiator tag sequence and the target sequence. Alternatively, the sequencing adaptors can be synthesized with the random differentiator tag sequences at their 3' end (as degenerate bases), so that only one ligation must be performed. Another method is to incorporate the differentiator tag sequence into a PCR primer, such that the primer structure is arranged with the common adaptor sequence followed by the random differentiator tag sequence followed by the PCR priming sequence (in 5' to 3' order). A differentiator tag sequence and adaptor sequence (which may contain the sequencing primer site) are incorporated as tags. Another method to incorporate the differentiator tag sequences is to synthesize them into a padlock probe prior to performing a gene capture reaction. The differentiator tag sequence is incorporated 3' to the targeting arm but 5' to the amplification primer that will be used downstream in the protocol. Another method to incorporate the differentiator tag sequences is as a tag on a gene-specific or poly-dT reverse-transcription primer. This allows the differentiator tag sequence to be incorporated directly at the cDNA level.

In some embodiments, at the incorporation step, the distribution of differentiator tag sequences can be assumed to be uniform. In this case, bias in any part of the protocol would change the uniformity of this distribution, which can be observed after sequencing. This allows the differentiator tag sequence to be used in any preparative process where the ultimate output is sequencing of many molecules in parallel.

Differentiator tag sequences may be incorporated into probes (e.g., MIPs) of a plurality when they are synthesized on-chip in parallel, such that degeneracy of the incorporated nucleotides is sufficient to ensure near-uniform distribution in the plurality of probes. It is to be appreciated that amplification of a pool of unique differentiator tag sequences may itself introduce bias in the initial pool. However, in most practical cases, the scale of synthesis (e.g., by column synthesis, chip based synthesis, etc.) is large enough that amplification of an initial pool of differentiator tag sequences is not necessary. By avoiding amplification or selection steps on the pool of unique differentiator tag sequences, potential bias may be minimized.

One example of the use of the differentiator tag sequences is in genome re-sequencing. Considering that the raw accuracy of most next-generation sequencing instruments is relatively low, it is crucial to oversample the genomic loci of interest. Furthermore, since there are two alleles at every locus, it is important to sample enough to ensure that both alleles have been observed a sufficient number of times to determine with a sufficient degree of statistical confidence whether the sample is homozygous or heterozygous. Indeed, the sequencing is performed to sample the composition of molecules in the originating sample. However, after multiple reads have been collected for a given locus, it is possible that due to bias (e.g., caused by PCR amplification steps), a large fraction of the reads are derived from a single originating molecule. This would skew the population of target sequences observed, and would affect the outcome of the genotype call. For example, it is possible that a locus that is heterozygous is called as homozygous, because there are only a few observations of the second allele out of many observations of that locus. However, if information is available on differentiator tag sequences, this situation could be averted, because the over-represented allele would be seen to also have an over-represented differentiator tag sequence (i.e., the sequences with the overrepresented differentiator tag sequence all originated from the same single molecule). Therefore, the sequences and corresponding distribution of differentiator tag sequences can be used as an additional input to the genotype-calling algorithm to significantly improve the accuracy and confidence of the genotype calls.

In some aspects, the disclosure provides methods for analyzing a plurality of to target sequences which are genetic loci or portions of genetic loci (e.g., a genetic locus of Table 1). The genetic loci may be analyzed by sequencing to obtain a genotype at one or more polymorphisms (e.g., SNPs). Exemplary polymorphisms are disclosed in Table 2. The skilled artisan will appreciate that other polymorphisms are known in the art and may be identified, for example, by querying the Entrez Single Nucleotide Polymorphism database, for example, by searching with a GeneID from Table 1.

TABLE 1

Target Nucleic Acids

Gene name	Gene ID	Description	Gene aliases	OMIM	RefSeqGene	Chromosome map position
CYP21 A2	1589	cytochrome P450, family 21, subfamily A, polypeptide 2	CAH1; CPS1; CA21H; CYP21; CYP21B; P450c21B; MGC150536; MGC150537; CYP21 A2	201910	NG_008337.1	6p21.3
ABCC8	6833	ATP-binding cassette, sub-family C (CFTR/MRP), member 8	HI; SUR; HHF1; MRP8; PHHI; SUR1; ABC36; HRINS; TNDM2; ABCC8	600509	NG_008867.1	11p15.1
ATRX	546	sub-family C	SHS; XH2; XNP; ATR2; SFM1; RAD54; MRXHF1; RAD54L; ZNF-HX; MGC2094; ATRX	300032	NG_008838.1	Xq13.1-q21.1
ARSA	410	(CFTR/MRP), member 8	MLD; ARSA	607574	NG_009260.1	22g13.31-qter; 22q13.33
PSAP	5660	Prosaposin	GLBA; SAP1; FLJ00245; MGC110993; PSAP	176801	NG_008835.1	10q21-q22
BTD	686	Biotinidase	BTD	609019	NG_008019.1	3p25
HLCS	3141	holocarboxylase synthetase (biotin-(proprionyl-Coenzyme A-carboxylase (ATP-hydrolysing)) ligase)	HCS; HLCS	609018	NC_000021.7	21q22.1; 21q22.13
BLM	641	Bloom syndrome, RecQ helicase-like	BS; RECQ2; RECQL2; RECQL3; MGC126616; MGC131618; MGC131620; BLM	604610	NG_007272.1	15q26.1
ASPA	443	aspartoacylase (Canavan disease)	ASP; ACY2; ASPA	608034	NG_008399.1	17pter-P13
CFTR	1080	cystic fibrosis transmembrane conductance regulator (ATP-binding cassette sub-family C, member 7)	CF; MRP7; ABC35; ABCC7; CFTR/MRP; TNR-CFTR; dJ760C5.1; CFTR	602421	NC_000007.12	7q31.2
ASS1	445	argininosuccinate synthetase 1	ASS; CTLN1; ASS1	603470	NG_011542.1	9q34.1
MMACHC	25974	methylmalonic aciduria (cobalamin deficiency) cb1C type, with homocystinuria	cb1C; FLJ25671; DKFZp564I122; RP11-291L19.3; MMACWC	609831	NC_000001.9	1p34.1
IKBKAP	8518	inhibitor of kappa light polypeptide gene enhancer in B-cclls, kinase complex-associated protein	FD; DYS; ELP1; IKAP; IKI3; TOT1; FLJ12497; DKFZp781H1425; IKBKAP	603722	NG_008788.1	9q31
FANCC	2176	Fanconi anemia, complementation group C	FA3; FAC; FACC; FLJ14675; FANCC	227645	NG_011707.1	9q22.3
GALK1	2584	galactokinase 1	GK1; GALK; GALK1	604313	NG_008079.1	17q24
GALT	2592	galactose-l-phosphate uridylyltransferase	GALT	606999	NC_000009.10	9p13

TABLE 1-continued

Target Nucleic Acids

Gene name	Gene ID	Description	Gene aliases	OMIM	RefSeqGene	Chromosome map position
GALE	2582	UDP-galactose-4-epimcrase	SDR1E1; FLJ95174; FLJ97302; GALE	606953	NG_007068.1	1p36-p35
GBA	2629	glucosidase, beta; acid (includes glucosylceramidase)	GCB; GBA1; GLUC; GBA	606463	NG_009783.1	1q21
GJB2	2706	gap junction protein, beta 2, 26 kDa	HID; KID; PPK; CX26; DFNA3; DFNB1; NSRD1; DFNA3A; DFNB1A; GJB2	121011	NG_008358.1	13q11-q12
GCDH	2639	glutaryl-Cocnzyme A dehydrogenase	GCD; ACAD5; GCDH	608801	NG_009292.1	19p13.2
G6PC	2538	glucose-6-phosphatase, catalytic subunit	G6PT; GSD1; GSD1a; MGCI63350; G6PC	232200	NG_011808.1	17q21
HBB	3043	hemoglobin, beta	CD113t-C; beta-globin; HBB	141900	NG_000007.3	11p15.5
BCKDHA	593	branched chain keto acid dehydrogenase E1, alpha polypeptide	MSU; MSUD1; OVD1A; BCKDE1A; FLJ45695; BCKDHA	608348	NC_000019.8	19q13.1-q13.2
BCKDHB	594	branched chain keto acid dehydrogenase E1, beta polypeptide	E1B; FLJ17880; dJ279A18.1; BCKDHB	248611	NG_009775.1	6q13-q15
DBT	1629	dihydrolipoamide branched chain transacylase E2	E2; E2B; BCATE2; MGC9061; DBT	248610	NG_011852.1	1p31
DLD	1738	dihydrolipoamide dehydrogenase	E3; LAD; DLDH; GCSL; PHE3; DLD	238331	NG_008045.1	7q31-q32
ACADM	34	acyl-Coenzyme A dehydrogenase, C-4 to C-12 straight chain	MCAD; ACAD1; MCADH; FLJ18227; FLJ93013; FLJ99884; ACADM	607008	NG_007045.1	1p31
MMAA	166785	methylmalonic aciduria (cobalamin deficiency) cb1A type	cb1A; MGC120010; MGC120011; MGC120012; MGC120013; MMAA	607481	NG_007536.1	4q31.22
MMAB	326625	methylmalonic aciduria (cobalamin deficiency) cb1B type	ATR; cb1B; MGC20496; MMAB	607568	NG_007096.1	12q24
MUT	4594	methylmalonyl Coenzyme A mutase	MCM; MUT	609058	NG_007100.1	6p12.3
MCOLN1	57192	mucolipin 1	ML4; MLIV; MST080; TRPML1; MSTP080; TRP-ML1; TRPM-L1; MCOLN1	605248	NC_000019.8	19p13.3-p13.2
ACTA1	58	actin, alpha 1, skeletal muscle	ACTA; ASMA; CFTD; MPFD; NEM1; NEM2; NEM3; CFTD1; CFTDM; ACTA1	102610	NG_006672.1	1q42.13
TPM3	7170	tropomyosin 3	TM3; TRK; NEM1; TM-5; TM30; TM30 nm; TPMsk3; hscp30; MGC3261; FLJ41118; MGC14582;	191030	NG_008621.1	1q21.2

TABLE 1-continued

Target Nucleic Acids						
Gene name	Gene ID	Description	Gene aliases	OMIM	RefSeqGene	Chromosome map position
			MGC72094; OK/SW-c1.5; TPM3			
TNNT1	7138	troponin T type 1 (skeletal, slow)	ANM; TNT; STNT; TNTS; F1198147; MGC104241; TNNT1	191041	NG_011829.1	19q13.4
NEB	4703	nebulin	NEM2; NEB177D; FLJ11505; FLJ36536; FLJ39568; FLJ39584; DKFZp686C1456; NEB	161650	NG_009382.1	2q22
SMPD1	6609	sphingomyelin phosphodiesterase 1, acid lysosomal	ASM; NPD; SMPD1	607608	NG_011780.1	11p15.4-p15.1
GLDC	2731	glycine dehydrogenase (decarboxylating)	GCE; NKH; GCSP; HYGN1; MGC138198; MGC138200; GLDC	238300	NC_000009.10	9p22
GCSH	2653	glycine cleavage system protein H (aminomethyl carrier)	GCE; NKH; GCSH	238330	NC_000016.8	16q23.2
AMT	275	aminomethyltransferase	GCE; NKH; GCST; AMT	238310	NC_000003.10	3p21.2-p21.1
OTC	5009	ornithine carbamoyltransferase	OCTD; MGC129967; MGC129968; MGC138856; OTC	300461	NG_008471.1	Xp21.1
PAH	5053	phenylalanine hydroxylase	PH; PKU; PKU1; PAH	612349	NG_008690.1	12g22-q24.2
DHPR	5860	quinoid dihydropteridine reductase	DHPR; PKU2; SDR33C1; FLJ42391; QDPR	612676	NG_008763.1	4p15.31
PTS	5805	6-pyruvoyltetrahydropterin synthase	PTPS; FLJ97081; PTS	261640	NG_008743.1	11g22.3-q23.3
PCCA	5095	propionyl Coenzyme A carboxylase, alpha polypeptide	PCCA	232000	NG_008768.1	13q32
PCCB	5096	propionyl Coenzyme A carboxylase, beta polypeptide	DKFZp451E113; PCCB	232050	NG_008939.1	3q21-q22
ACADS	35	acyl-Coenzyme A dehydrogenase, C-2 to C-3 short chain	SCAD; ACAD3; ACADS	606885	NG_007991.1	12g22-qter
DHCR7	1717	7-dehydrocholesterol reductase	SLOS; DHCR7	602858	NC_000011.8	11q13.2-q13.5
SMNT	6606	survival of motor neuron 1, telomeric	SMA; SMN; SMA1; SMA2; SMA3; SMA4; SMA @; SMNT; BCD541; T-BCD541; SMN1	600354	NG_008691.1	5q13
HEXA	3073	hexosaminidase A (alpha polypeptide)	TSD; MGC99608; HEXA	606869	NG_009017.1	15g23-q24
MYO7A	4647	myosin VIIA	DFNB2; MYU7A; NSRD2; USH1B; DFNA11; MYOVIIA; MYO7A	276903	NG_009086.1	11q13.5
USH1C	10083	Usher syndrome 1 C (autosomal recessive, severe)	PDZ73; AIE-75; DFNB18; PDZ-45; PDZ-	605242	NC_000011.8	11p15.1-p14

TABLE 1-continued

Target Nucleic Acids						
Gene name	Gene ID	Description	Gene aliases	OMIM	RefSeqGene	Chromosome map position
			73; NY-CO-37; NY-CO-38; ush1cpst; PDZ-73/NY-CO-38; USH1C			
CDH23	64072	cadherin-like 23	USH1D; DFNB12; FLJ00233; FLJ36499; KIAA1774; KIAA1812; MGC102761; DKFZp434P2350; CDH23	605516	NG_008835.1	10g21-q22
PCDH15	65217	protocadherin 15	USH1F; DFNB23; DKFZp667A1711; PCDH15	605514	NG_009191.1	10q21.1
SANS	124590	Usher syndrome 1G (autosomal recessive)	SANS; ANKS4A; FLJ33924; USH1G	607696	NG_007882.1	17q25.1
ARX	170302	aristaless related homeobox	ISSX; PRTS; MRX29; MRX32; MRX33; MRX36; MRX38; MRX43; MRX54; MRX76; MRX87; MRXS1; ARX	300382	NG_008281.1	Xp21
OPHN1	4983	oligophrenin 1	OPN1; MRX60; OPHN1	300127	NG_008960.1	Xq12
JAR1DIC	8242	lysine (K)-specific demethylase 5C	MRXJ; SMCX; MRXSJ; XE169; JARID1C; DXS1272E; KDM5C	314690	NG_008085.1	Xp11.22-p11.21
FTSJ1	24140	FtsJ homolog 1 (*E. coli*)	JM23; MRX9; SPB1; TRM7; CDLIV; MRX44; FTSJ1	300499	NG_008879.1	Xp11.23
SLC6A8	6535	solute carrier family 6 (neurotransmitter transporter, creatine), member 8	CRT; CT1; CRTR; MGC87396; SLC6A8	300036	NC_000023.9	Xq28
DLG3	1741	discs, large homolog 3 (*Drosophila*)	MRX; MRX90; NEDLG; NE-Dlg; SAP102; SAP-102; KIAA1232; DLG3	300189	NC_000023.9	Xq13.1
TM4SF2	7102	letraspanin 7	A15; MXS1; CD231; MRX58; CCG-B7; TM4SF2; TALLA-1; TM4SF2b; DXS1692E; TSPAN7	300096	NG_009160.1	Xp11.4
ZNF41	7592	zinc finger protein 41	MRX89; MGC8941; ZNF41	314995	NG_008238.1	Xp11.23
FACL4	2182	acyl-CoA synthetase long-chain family member 4	ACS4; FACL4; LACS4; MRX63; MRX68; ACSL4	300157	NG_008053.1	Xq22.3-q23
PQBP1	10084	polyglutamine binding protein 1	SHS; MRX55; MRXS3;	300463	NC_000023.9	Xp11.23

TABLE 1-continued

Target Nucleic Acids

Gene name	Gene ID	Description	Gene aliases	OMIM	RefSeqGene	Chromosome map position
			MRXS8; NPW38; RENS1; PQBP1			
PEX1	5189	peroxisomal biogenesis factor 1	ZWS1; PEX1	602136	NG_008341.1	7q21.2
PXMP3	5828	peroxisomal membrane protein 3, 35 kDa	PAF1; PEX2; PMP3; PAF-1; PMP35; RNF72; PXMP3	170993	NG_008371.1	8q21.1
PEX6	5190	peroxisomal biogenesis factor 6	PAF2; PAF-2; PXAAA1; PEX6	601498	NG_008370.1	6p21.1
PEX10	5192	peroxisomal biogenesis factor 10	NALD; RNF69; MGC1998; PEX10	602859	NG_008342.1	1p36.32
PEX12	5193	peroxisomal biogenesis factor 12	PAF-3; PEX12	601758	NG_008447.1	17q12
PEX5	5830	peroxisomal biogenesis factor 5	PXR1; PTS1R; PTS1-BP; FLJ50634; FLJ50721; FLJ51948; PEX5	600414	NG_008448.1	12p13.31
PEX26	55670	peroxisomal biogenesis factor 26	FLJ20695; PEX26M1T; Pex26pM1T; PEX26	608666	NG_008339.1	22q11.21

The mutations listed in Table 2 are documented polymorphisms in several disease-associated genes (CFTR is mutated in cystic fibrosis, GBA is mutated in Gaucher disease, ASPA is mutated in Canavan disease, HEXA is mutated in Tay Sachs disease). The polymorphisms are of several types: insertion/deletion polymorphisms which will cause frameshifts (and thus generally interrupt protein function) unless the insertion/deletion length is a multiple of 3 bp, and substitutions which can alter the amino acid sequence of the protein and in some cases cause complete inactivation by introduction of a stop codon.

TABLE 2

Non-Limiting Examples of Polymorphism

Gene name	GeneID	SNP ID	Mutation	SEQ ID NO:
CFTR	1080	rs63500661	TCACATCACCAAGTTAAAAAAAAAAA[A/G]G GGGCGGGGGGGCAGAATGAAAATT	1
CFTR	1080	rs63107760	AAACAAGGATGAATTAAGTTTTTTTT[-/T] AAAAAAGAAACATTTGGTAAGGGGA	2
CFTR	1080	rs62469443	ATCACCAAGTTAAAAAAAAAAAAGGG[A/G]C GGGGGGGCAGAATGAAAATTGCAT	3
CFTR	1080	rs62469442	CTATTGAACCAGAACCAAACAGGAAT[A/G]C CATAGCATTTTGTAAACTAAACTG	4
CFTR	1080	rs62469441	CAGGAGTTCAAGACCAGCCTACTAAA[A/C]C ACACACACACACACACACACACAC	5
CFTR	1080	rs62469439	GATTAAATAATAGTGTTTATGTACCC[C/G]GC TTATAGGAGAAGAGGGTGTGTGT	6
CFTR	1080	rs62469438	ATTGTTATCTTTTCATATAAGGTAAC[A/T]GA GGCCCAGAGAGATTAAATAACAT	7
CFTR	1080	rs62469437	TAATTTTAATTAAGTAAATTTAATTG[A/G]TA GATAAATAAGTAGATAAAAAATA	8
CFTR	1080	rs62469436	GTATAAAAAAAAAAAAAAAAAAAGTT[A/T]G AATGTTTTCTTGCATTCAGAGCCT	9

TABLE 2-continued

Non-Limiting Examples of Polymorphism

Gene name	GeneID	SNP ID	Mutation	SEQ ID NO:
CFTR	1080	rs62469435	ATACTAAAAATTTAAAGTTCTCTTGC[A/G]ATATATTTTCTTAATATCTTACATC	10
CFTR	1080	rs62469434	TGCTGGGATTACAGGCGTGAGCCACC[A/G]CGCCTGGCCTGATGGGACATATTTT	11
CFTR	1080	rs62469433	CTACAATATAAGTATAGTATTGCAAA[A/C]CCATCAGGAAGGGTGTTAACTATTT	12
CFTR	1080	rs61763210	GTTGTCTCCAAACTTTTTTTCAGGTG[-/AGA]AGGTGGCCAACCGAGCTTCGGAAAG	13
CFTR	1080	rs61720488	TTTTTTCATAAAAGATTATATAAAGG[A/C]TATTGCTTTTGAATCACAAACACTA	14
CFTR	1080	rs61481156	ATCTAGTGAGCAGTCAGGAAAGAGAA[C/T]TTCCAGATCCTGGAAATCAGGGTTA	15
CFTR	1080	rs61443875	TAGAGTATAAAAAAAAAAAAAAAAAA[-/A]GTTTGAATGTTTTCTTGCATTCAGA	16
CFTR	1080	rs61312222	TGCAAATGCCAACTATCAAAGATATT[C/G]GAGTATACTGTCAATAAACTTCATA	17
CFTR	1080	rs61159372	TCCTCAACAGTTAGAAACAATATTTT[C/G]AGTGATTTCCCATGCCAACTTTACT	18
CFTR	1080	rs61094145	TTTTTGGTATTGTTGTTAAATAAGTG[A/G]GAATTCAATACAGTATAATGTCTGT	19
CFTR	1080	rs61086387	CTTGAAATCGGATATATATATATATA[-/TGTATATATATATATATATATATATATATATACATATATATATATATA]GTATTATCCCTGTTTTCACAGTTTT	20
CFTR	1080	rs60996744	AGAGGGGCTGTGAAGGACACCAAGGA[A/G]GAGACTAAGAGCCAGGAGGGAAAAC	21
CFTR	1080	rs60960860	TAGAGTTTATTAGCTTTTACTACTCT[A/G]CTTAGTTACTTTGTGTTACAGAATA	22
CFTR	1080	rs60923902	ACTAGTGATGATGAGCTTCTTTTCAT[-/AT]GTTTGTTGGCTGCATAAATGTCTTC	23
CFTR	1080	rs60912824	GCAGAGAAAAGAGGGGCTGTGAAGGA[C/G]ACCAAGGAGGAGACTAAGAGCCAGG	24
CFTR	1080	rs60887846	TTCAGAGGTCTACCACTGGTGCATAC[G/T]CTAATCACAGTGTCGAAAATTTTAC	25
CFTR	1080	rs60793174	AAGAAAGAGCAAAAGAGGGCAAACTT[C/T]TCATACATTTTTGATGTCGAAACCA	26
CFTR	1080	rs60788575	CCTAAAGTTTAAAAAGAAAAAAAAAA[-/A]GGAAGAAGGAATTAAAAATCCAAAG	27
CFTR	1080	rs60760741	GTGTGTGTGTGTATATATATATATAT[A/T]TATATATTTTTTTTTTCCTGAGCCA	28
CFTR	1080	rs60456599	AAACTGTTGATGTTTTCATTTATTTA[C/G]ATCATTGGAAAACTTTAGATTCTAG	29
CFTR	1080	rs60363249	TTTATCCATTCTTAACCAGAACAGAC[A/G]TTTTTTCAGAGCTGGTCCAGGAAAA	30
CFTR	1080	rs60355115	TTGAAATCGGATATATATATATATAT[A/G]TATATATATATATATATATATATAT	31
CFTR	1080	rs60308689	TAGTTTTTTATTTCCTCATATTATTT[-/T]CAGTGGCTTTTTTCTTCCACATCTTT	32
CFTR	1080	rs60271242	ACATAGTTCTCAGTGGTACAACTACA[A/G]GTGATTTCTCTTTTCTTATTTCTGG	33

TABLE 2-continued

Non-Limiting Examples of Polymorphism

Gene name	GeneID	SNP ID	Mutation	SEQ ID NO:
CFTR	1080	rs60010318	AGAGCAATGGCATCCCTTGTCTTGTG[C/T]TATACAGGATGCAGCAATTTATAGG	34
CFTR	1080	rs59961323	TTCTGTCTACATAAGATGTCATACTA[A/G]ATTATCTTTTCCAGCATGCATTCAG	35
CFTR	1080	rs59961270	CAGGGTGGCATGTTAGGCAGTGCTTA[A/G]AATAAATGAGTTGGTTATACAAGTA	36
CFTR	1080	rs59837506	AGGACACACACACACACACACACACA[-/CA]TGCACACACATTTAAATAGATGCAT	37
CFTR	1080	rs59572090	TAAAAAATTGGTATAATGAAATTGCA[C/T]TTGTAGTCTTTGGACATTTAAATCC	38
CFTR	1080	rs59548252	TTTCAATACTTAAGAGGTACGCAGAG[A/G]AAAGAGGGGCTGTGAAGGACACCAA	39
CFTR	1080	rs59519859	CAGCAATGAATATTTTGAGGCTGAGG[C/T]GCTGAGGGGTAAAATTGCAGCCTGG	40
CFTR	1080	rs59509837	TTATGGTTTATATTTTGTGTCTTCT[-/CTTT]AACACATCTTTTCTAGCAGAATTCA	41
CFTR	1080	rs59417037	GTATTTTAGTTTTTTTTTTTTGTTTG[-/T]TTTGTTTTGTTTTGTTTTGTTTTTG	42
CFTR	1080	rs59159458	TGGGTGACTCCATTTTTACTTTTAGT[C/T]TGGTCTGTTGAGGCCTCGTGAGAGA	43
CFTR	1080	rs59048119	TATTTTCATGTATTTTAGTTTTTTTTT[-/TTTT]GTTTGTTTTGTTTTGTTTTGTTTTG	44
CFTR	1080	rs58970500	GTGTGTGTGTATATATATATATATAT[A/T]TATATTTTTTTTTTCCTGAGCCAAA	45
CFTR	1080	rs58942292	AACCTATTAGCATGTCTGGCAGAAAA[-/A]TAGATACTTAATAAATTTCTTAAAT	46
CFTR	1080	rs58917054	GAGGCTTAGACAGTTTAAGTAACTCA[A/G]GCATGGTTACACAACTAGCTAGGGC	47
CFTR	1080	rs58837484	GTGTGAGTATTATGAGACCATATGTT[A/G]GGAGATTTTATTTGGTATTGAGGAT	48
CFTR	1080	rs58829491	GAAACCCCACCCCTTCTATAGTTTTC[C/T]CTTTAATATTTACAATGGAACCATT	49
CFTR	1080	rs58805195	CATATATATATAGTGTGTGTGTGTGT[A/G]TATATATATATATATATATATTTTT	50
GBA	2629	rs60866785	CGAGCGAGAGAGAGAGAGAGAGAGAG[-/AG]GAGCCGGCGCGAGAACTACGCATGC	51
GBA	2629	rs60239603	GGCAGGTAATATCTAGTACCTTACTT[A/T]TATTTCCTGAGCACATTCTACATTT	52
GBA	2629	rs56310840	GGCCAGGAATGGGAGTGCTTAGGTGC[A/G]GAGGTGGCACTGTTCCCGCAGCTGC	53
GBA	2629	rs41264927	GAAAACTCCATCCCCTCAGGGTCAT[C/T]AGATGAAGAGAAGACCACAGGGGTT	54
GBA	2629	rs41264925	TGTAGGTAAGGGTCACATGTGGGAGA[C/G]GCAGCTGTGGGTAGGTCAGCCCTGT	55
GBA	2629	rs36024691	CCAAGAAGGCGCCATTACACTCCAGC[-/C]TGGGCGACAGGGCGAGACTCCCTCA	56
GBA	2629	rs36024092	TGCCACACCCAGCTAATTTGTGTGTG[-/G]TATGTGTGTGTATGTATGTGTGTGT	57

TABLE 2-continued

Non-Limiting Examples of Polymorphism

Gene name	GeneID	SNP ID	Mutation	SEQ ID NO:
GBA	2629	rs35682967	GTTCCTCCAGTAATTTTTTTTTTTTT[-/T]GGTTTTGAGACAGAGTCTTGCCCTG	58
GBA	2629	rs35033592	ATCATGCCCAGATAATTTTTTTTTTT[-/T]GTATTTTAGTAGACACAGGGTTTCA	59
GBA	2629	rs34732744	CGAGCGAGAGAGAGAGAGAGAGAGAG[-/AG]GAGCCGGCGCGAGAACTACGCATGC	60
GBA	2629	rs34620635	CCTGTGAGGGGCACATTCCTTAGTAG[-/C]TAAGGAGTTGGGGGTGTGAAGATCC	61
GBA	2629	rs34302637	ACAGGCTACTGGCTGGGCCCAGGCAA[-/A]GGGGGCCTTGGCAGGAAAAGTTCCT	62
GBA	2629	rs33949225	GCGAGAGAGAGAGAGAGAGAGAGAGG[-/AG]AGCCGGCGCGAGAACTACGCATGCG	63
GBA	2629	rs28678003	AAGAAGAAAAATAAAAAGAAAGTGGG[C/T]CAGACCGAGAGAACAGGAAGCCTGA	64
GBA	2629	rs28559737	AAGGACAAAGGCAAAGAGACAAAGGC[G/T]CAACACTGGGGGTCCCCAGAGAGTG	65
GBA	2629	rs28373017	TACCTAGTCACTTCCTGCCTCCATGG[C/T]GCAAAAGGGGATGGGTGTGCCTCTT	66
GBA	2629	rs12752133	CTCTTCCGAGGTTCCACCCTGAACAC[C/T]TTCCTGCTCCCTCGTGGTGTAGAGT	67
GBA	2629	rs12747811	TTCTGACTGGCAACCAGCCCCACTCT[C/T]TGGGAGCCCTCAGGAATGAACTTGC	68
GBA	2629	rs12743554	gctcagcctcccaggctggagtgcag[A/T]ggcgcgatctcggctcaccgcaacc	69
GBA	2629	rs12041778	CATGAACCACATCAAATGAGATTTAG[C/T]GGGAGTGGCACACACAGTCATGACC	70
GBA	2629	rs12034326	AAGCAGCCCTGGGGAGTCGGGGCGGG[A/G]CCTGGATTGGAAAAGAGACGGTCAC	71
GBA	2629	rs11558184	CTCCAAGTTCTGGGAGCAGAGTGTGC[A/G]GCTAGGCTCCTGGGATCGAGGGATG	72
GBA	2629	rs11430678	GTTCCTCCAGTAAttttttttttttt[-/G/T]gttttgagacagagtcttgccctgt	73
GBA	2629	rs11264345	CTAGTACCTTACTTCCCTCAAGTTCA[A/T]TCATCTCACAGATATTTCCTGAGCA	74
GBA	2629	rs10908459	aattagccgtgcgtggtggcgggtgc[C/T]tgtaatcccacgtacttgggaggct	75
GBA	2629	rs10796940	CCATGGCCAGCCGGGGAGGGGACGGG[A/C]ACACACAGACCCACACAGAGACTCA	76
GBA	2629	rs10668496	agcgagagagagagagagagagagag[-/AG]gagCCGGCGCGAGAACTACGCATGC	77
GBA	2629	rs7416991	CGTAGCAGTTAGCAGATGATAGGCGG[C/G/T]GAAATCTTATTTCACAGGGCATTAA	78
GBA	2629	rs4024049	CTGGCCCTGGTGACAGTGGGGCTGTG[C/T]GTGGGGCCAGAGCCTTCTCAGAGGT	79
GBA	2629	rs 4024048	CAGATACTGGCCCTGGTGACAGTGGG[A/G]CTGTGCGTGGGGCCAGAGCCTTCTC	80
GBA	2629	rs 4024047	GACAGATACTGGCCCTGGTGACAGTG[G/T]GGCTGTGCGTGGGGCCAGAGCCTTC	81

TABLE 2-continued

Non-Limiting Examples of Polymorphism

Gene name	GeneID	SNP ID	Mutation	SEQ ID NO:
GBA	2629	rs3841430	GGCTCctctctctctctctctctctc[-/TC]gctcgctctctcgctctctcgctct	82
GBA	2629	rs3754485	GTTTCAGACCAGCCTGGCCAACATAG[C/T]GAAACCCCATCTCTACTAAAAATAA	83
GBA	2629	rs3205619	AGTGGGCGATTGGATGGAGCTGAGTA[C/T]GGGGCCCATCCAGGCTAATCACACC	84
GBA	2629	rs2990227	CCGGGCTCCGTGAATGTTTGTCACAT[C/G]TCTGAAGAACGTATGAATTACATAA	85
GBA	2629	rs2990226	GAATCCCAACCCCGACGCTCGTCGCC[C/G]GGCTCCGTGAATGTTTGTCACATGT	86
GBA	2629	rs2990225	GCGAATCCCAACCCCGACGCTCGTCG[C/T]CGGGCTCCGTGAATGTTTGTCACAT	87
GBA	2629	rs2990224	TGGGCAGAAGTCAGGGTCCAAAGAAA[G/T]GGCAAAGAAAAGTGTcagtggctca	88
ASPA	443	rs63751297	TAAGAAAGACGTTTTTGATTTTTTTC[A/G]GACTTCTCTGGCTCCACTACCCTGC	89
ASPA	443	rs62071301	CTGATTCCTGGCCAGGAGCGGTGGCT[C/T]ACGCCTGTAATCCCAGCGCTTTGGG	90
ASPA	443	rs62071300	TAAAAATGCTGATTCCTGGCCAGGAG[C/T]GGTGGCTCACGCCTGTAATCCCAGC	91
ASPA	443	rs62071299	TTTAAAAATGCTGATTCCTGGCCAGG[A/C]GCGGTGGCTCACGCCTGTAATCCCA	92
ASPA	443	rs62071297	CAAGACCTGTCAAAGATCTGAGAAAT[A/T]TTACCCGACTTACAAGCTAACCATT	93
ASPA	443	rs61697033	ACTGTAATAAGTGCTGTAAAAGAAAT[A/G]CACAAAATAATATAGCAGAGGGTAT	94
ASPA	443	rs60743592	CTTGAGGTCAGGAGTTCAAGACCAGT[C/T]TGGGCAACATGGGGAAAACCTTGTC	95
ASPA	443	rs60666840	AGGTTGCAGTGAGCCGAGATCATGCC[A/G]TTGCACTCCAGCCGGGGCAACAAAA	96
ASPA	443	rs60147514	ACAAGTGTCTTGAAATTATCTGTGAT[C/T]TGCTATAGAGCAATACTTTTGTAAA	97
ASPA	443	rs59930743	GTGGGTATATGCAGCTCTATGCACTA[C/T]CTGCTCATTTATTTGGTAAATCTAA	98
ASPA	443	rs59690349	TGTGTGTGTGCGTGTGTGTGTGTG[-/TGTGTGTG]ATCATAAGAGTGGCTGCAGCAAACT	99
ASPA	443	rs59676360	AGTCTGGAGTGCAATGGTGCAATCTC[A/G]GCTCACTGCAGCCTCCACCTCCGGG	100
ASPA	443	rs59335404	CTCCTAATGGATATTTCCTAAATTTT[G/T]CTGAACAGAATTTAACTTGAGCTGG	101
ASPA	443	rs58879097	ATTTAAAAATGGATTTCTAGAAAAAC[A/G]ATCACATACTTGAATATTTTAGCAA	102
ASPA	443	rs58686774	CTATAAATGGGTAGCATGAGGGATTC[A/G]AGGAGGTGGCTGAAAGAAGCACGTA	103
ASPA	443	rs57511162	AAGAAACCAAGCATAGTAGAGTGTTA[A/G]AAAACCAAAGCAACTAAACAACTGT	104
ASPA	443	rs55859596	CGGGGCTCAGAACTTGTAACAGAAAA[A/T]TAAAATATACTCCACTCAAGGGAAT	105

TABLE 2-continued

Non-Limiting Examples of Polymorphism

Gene name	GeneID	SNP ID	Mutation	SEQ ID NO:
ASPA	443	rs55742972	TACTACACTTCACGGATACTGTACTT[-/GTACTT]TTTTTCCAAATTGAAGGTTTTTGGC	106
ASPA	443	rs55640436	TTGTTTTTGTTTTTGTTTTTGTTTTT[-/GTTTTTGTTTTT]TGAGATGGAGTCTCGCTCTGTCGCC	107
ASPA	443	rs36225687	TTTGCCTTACTACACTTCACGGATAC[-/TGTACT]TGTACTTTTTTTCCAAATTGAAGGT	108
ASPA	443	rs36051310	GAGGTGGCTGAAAGAAGCACGTATCC[-/C]TGATGGCATGGTTGCGGGTTATATG	109
ASPA	443	rs36034906	GAGAAAAGCAGTTCCTGGAACACCCC[-/C]ACCCCTTAACCCCTTATCTCTGCTT	110
ASPA	443	rs36033666	TTACATATGTATACATGTGCCATGTT[-/T]GGTGTGCCGCACCCATTAACTCGTC	111
ASPA	443	rs35730123	CTTTTTCCAGATTTTTTTTTTTTTTT[-/T]GAGACAGAGTTTCACTCTTGTTGCC	112
ASPA	443	rs35629100	TTTGGAAATCTTAAGCTTTTATTTGG[-/G]TGTCACAGAGAAACAGGATCTGTAT	113
ASPA	443	rs35614631	TACTTTAAGTTTTAGGGTACATGTGC[-/A]CCATGTGCAGGTTTGTTACATATGT	114
ASPA	443	rs35225782	ATTCATGACCAGCCACATAAATGCAC[-/A]GTATTACTTCGCAAGCATGCCAATG	115
ASPA	443	rs35178659	GTGCACTAGAATTAGCTAAAGTGGGG[-/G]AAAAAAAGATGCATTTGATGGTCTA	116
ASPA	443	rs35095578	AACCTCCACCTCCCAGGTTCAAGAGA[-/A]TTCTCCTGCCTCAGCCTCCCAAGTA	117
ASPA	443	rs35002210	CCTCCCTGTGATCCGAAGTAGCAGAC[A/G]TACTTAACTTCCATGGTGGATTGTT	118
ASPA	443	rs34744839	AAAACATTATTATATCTAGAAAAAAA[-/A]TGTATCTTAACCATTGTGGGAAGTG	119
ASPA	443	rs34680506	TTGAAGGTAAAATCATAGGGAGTTGG[-/G]AGCTGTCCTCTTGCGCTGAATCAGT	120
ASPA	443	rs34365618	ACTTGTGGCCTTTTTGGAGAGGTTAG[-/CA]ACTCTGAAAACTCTGTCCCTGGACC	121
ASPA	443	rs34275920	GAAGGAGAAAAAGAGAGGAAATAAGT[-/T]AAAATAATAAACACAATTAATAAAG	122
ASPA	443	rs34109510	TGTATACATGTGCCATGTTGGTGTGC[C/T]GCACCCATTAACTCGTCATTTAGCA	123
ASPA	443	rs34054576	TCACCTGTCACCTCCTATAGAACTTT[-/C]CCCTGACCCTCCTCTATAGCATTAA	124
ASPA	443	rs34015272	ATAAATGATCATCATTCACAGTAGGG[-/G]TTTTGTTTTGTTTTTTTTCTGGAAA	125
ASPA	443	rs34002091	ACAGACATATCTACAAACACACTTTT[-/T]CACATATTTGTGTAAGTCATTTATG	126
ASPA	443	rs28940574	AAAGACAACTAAACTAACGCTCAATG[A/C]AAAAAGTATTCGCTGCTGTTTACAT	127
ASPA	443	rs28940279	TACCGTGTACCCCGTGTTTGTGAATG[A/C]GGCCGCATATTACGAAAAGAAAGAA	128

TABLE 2-continued

Non-Limiting Examples of Polymorphism

Gene name	GeneID	SNP ID	Mutation	SEQ ID NO:
ASPA	443	rs17850703	CAGGGCTGGAGGTAAAACCATTTATT[A/G]CT AACCCCAGAGCAGTGAAGAAGTG	129
ASPA	443	rs17222495	TTCTTCATTGCCTATTGAAGAGAGAG[C/T]GG AATGCTTTGGTTGCCAGATATGG	130
ASPA	443	rs17175228	CACAAGATCTCATTACTCAGGAGCTG[C/T]CC AAGTGTCTAATGTACTTAGTTAA	131
ASPA	443	rs16953074	TTCTGTGTAACATTTCATTTAAGCAA[A/G]GG ATTCGGCAAATCAAAAATTGTCA	132
ASPA	443	rs16953070	TAAAACGTATTGAAGGTATTATTGAC[G/T]CT GTTGAAGCAAAGAGAACAAAACA	133
HEXA	3073	rs62022858	ATCTGCTCTTCCAGTTGGATGACAAG[C/T]CT TGCTGTCTAACACCTGCTGCAGA	134
HEXA	3073	rs62022857	CCATTTTTTGTTGTATTTTTTTTTTC[C/T]TGAA TACTTTTTATCGCAGTTGGTT	135
HEXA	3073	rs62017872	CCCTGTCTCTAAAAGAAAAAAAAAAA[A/G]A AAAAAAAAAGAAAACAAAACCCAA	136
HEXA	3073	rs62017871	AGTGGCTCCAAAAAGGTCATGGAACC[C/T]CT TGAGGATGATGCAAATTGACTCT	137
HEXA	3073	rs61662730	TAAAGTTACTTTTCTTTTATTGACTT[C/T]CCC TTATTTTTTAACCTTATGCTTT	138
HEXA	3073	rs61329913	CAGAGTTAAAAAAAAAAAAAAAAAAA[-/A] GGAAGTAGCAGCAACAGCTTGGAAA	139
HEXA	3073	rs60920713	GTTGCCCAGGGTTGAGTGCAGAGGCA[C/T]AT TTGGCTCACAGCAACCTCTGCC	140
HEXA	3073	rs60783213	AAGGCTTTTTTTTTTTTTTTTTTTTT[-/T TTT]GAGACAGAGTCTTGCTGTGTCACCC	141
HEXA	3073	rs60644867	GCCTACATTCTGCAAAGAGGAGGGAA[C/G]A TTCACAGCTCCATACTTGAACCCT	142
HEXA	3073	rs60288568	CCAAAGGAGAATAGCTCTAGGGGAGG[C/G]A GGTGGATGAGTATGCATGGGGGAG	143
HEXA	3073	rs59888548	GACTCCATCTCAAAAAAAAAAAAAAA[-/A] TGCAGTCTAATGGCAGAATTAGACT	144
HEXA	3073	rs59733856	TTATTTATTTATTTATTTATTTTTGA[A/G]ACA GGGTCTCTGTTGTCCAGGCTGG	145
HEXA	3073	rs59427837	TTTTGAGGCAGGGTCTCACTCTGTTG[C/T]CC AGGGTTGAGTGCAGAGGCACATC	146
HEXA	3073	rs59171976	CGCCTTGCGAAGGCCCCACAGCTTGC[C/T]TG TGACAAACGTTCATAGGCAAATG	147
HEXA	3073	rs58706602	GGAGGTCTGTACAAAGCACCACCTAC[C/T]TC ATGGGTCAGTTTCCACAGCAGAA	148
HEXA	3073	rs58696963	GAATCTTATAATTCACTGTGTACCTC[-/C CTC]TGTTTCATATTTTCGCAATTGAACT	149
HEXA	3073	rs58610850	AACATAGTATCTAATATAGCTTTACA[C/T]CC AAAGCCAAAATATGAATACACTG	150
HEXA	3073	rs58016062	TTGTTTTGTTTTGTTTGGGGGGGGGG[-/G] TTGTTTTTCTGAGAGGGAGTCTTGC	151
HEXA	3073	rs57733983	CATACCAAAGGGCAGCTGGAGGGATAC[C/T]A GACGGAAGTCATGTGGAGAGTGAA	152

TABLE 2-continued

Non-Limiting Examples of Polymorphism

Gene name	GeneID	SNP ID	Mutation	SEQ ID NO:
HEXA	3073	rs57476645	CAGGTGTGAGCCACCACGACCACCAA[A/T]TTAGCTCTTTTTACTCCTTCCCTTC	153
HEXA	3073	rs56870003	AGTGGTAGCTGATTTTGCTTCTGGAT[A/C]CTTTGCCACCTTCCCACTCTTTAAT	154
HEXA	3073	rs56338339	AAAGACCTGTTTCTTAAAAAAAAAAA[-/AGAAAAAAAAAAA]GAAAGAAAAGAAAAGAAAAAAACAG	155
HEXA	3073	rs55995352	TAAAAAATCTTTCAATGAGGAGATGT[C/T]CCCAGAGCAAGACAGCTGTAGGATG	156
HEXA	3073	rs55860138	AAAAGAAAAAAAAAAAAAAAAAAAAA[-/A]GAAAACAAAACCCAAACCCATAAAG	157
HEXA	3073	rs55743646	CCTGTCTCTAAAAGAAAAAAAAAAAA[A/G]AAAAAAAAAGAAAACAAAACCCAAA	158
HEXA	3073	rs55665666	GTTATCATAGAAAAATATCACACTCT[-/GT]CTGTATCCCCACTTCCAGAAACTGT	159
HEXA	3073	rs36106892	CAGGAGCTCATAGAATTACATACAAT[-/C]TTTTTTTTTTTTTTTTGAGACAGCG	160
HEXA	3073	rs36091525	TTGAGAATCTTATAATTCACTGTGTA[-/CCTC]CCTCTGTTTCATATTTTCGCAATTG	161
HEXA	3073	rs35949555	CCACTACCACAGTGCCTAGAGAACAA[C/T]ATGTGTTTAATAATATTTAAATAAT	162
HEXA	3073	rs35827424	CCCTGTCTCTAAAAGAAAAAAAAAAA[-/A]AAAAAAAAAAGAAAACAAAACCCAA	163
HEXA	3073	rs35729578	CCATTATATCATTCATTTCCCACTCA[-/T]TTTCTTCATTCCAACCAAGATATAT	164
HEXA	3073	rs35649102	TCCGTCTCAAAAAAAAAAAAAAAAAG[-/A]GAAAGGAATTATTCTCATGTATACA	165
HEXA	3073	rs35118677	CTGGGGCAGTTAAAAAGAAAAACAAA[-/C]CCCTGGTCCCTGCCCTTGAGGAGAT	166
HEXA	3073	rs35005352	CTCCAGGGTCCCATTCCAGGACCACA[-/C]GCCTGCTACCTCTGCAGCTCACTCA	167
HEXA	3073	rs34736306	GGATTGACATATACCAGTTAGACGGA[-/T]TTTTTTTTTCCATAAACCAGGCTCA	168
HEXA	3073	rs34607939	ACAAATAATTACTACATATCTACAAC[A/G]TTCCAGATACAGAAGAAATGGCCAA	169
HEXA	3073	rs34496117	TAAACACACTTGAAACATCATATAAA[-/ATG]ATATTACTACAAGACTTAACCGTAA	170
HEXA	3073	rs34300017	ACACAGGTAATCCATGTTTATTATAG[-/A]AAAATGCCACATTACTCTTTATTGA	171
HEXA	3073	rs34206496	AGTTATCATAGAAAAATATCACACTC[-/TG]TCTGTATCCCCACTTCCAGAAACTG	172
HEXA	3073	rs34110830	AATGAACTTACAGGAAGGTAATATAT[-/G]GGAAATAAACATCTTATTGAATTTA	173
HEXA	3073	rs34093438	GGACCCCTGAAAGGCACAAGACACCC[-/T]TTCAGGTTCACACTTCCTGAAAGCT	174
HEXA	3073	rs34085965	CCACCAATCACCAGAGCCTTCTGCTC[A/G]GGGGTACCTGAGGGAAAACAAGCAA	175
HEXA	3073	rs34004907	AAAGACTGAAAAAACATTCATAACTA[-/T]TTTTCTTGTTATCCTCGGAAATGTC	176

TABLE 2-continued

Non-Limiting Examples of Polymorphism

Gene name	GeneID	SNP ID	Mutation	SEQ ID NO:
HEXA	3073	rs28942072	TATCTTCATCTTGGAGGAGATGAGGT[C/T]GA TTTCACCTGCTGGAAGTCCAACC	177
HEXA	3073	rs28942071	TTGCCTATGAACGTTTGTCACACTTC[C/T]GCT GTGAGTTGCTGAGGCGAGGTGT	178
HEXA	3073	rs28941771	GCTTGCTGTTGGATACATCTCGCCAT[C/T]AC CTGCCACTCTCTAGCATCCTGGA	179
HEXA	3073	rs28941770	CCGGGGCTTGCTGTTGGATACATCTC[G/T]CC ATTACCTGCCACTCTCTAGCATC	180

3. Nucleic Acid Target Length Evaluation:

In some embodiments, aspects of the invention relate to methods for detecting nucleic acid deletions or insertions in regions containing nucleic acid sequence repeats.

Genomic regions that contain nucleic acid sequence repeats are often the site of genetic instability due to the amplification or contraction of the number of sequence repeats (e.g., the insertion or deletion of one or more units of the repeated sequence). Instability in the length of genomic regions that contain high numbers of repeat sequences has been associated with a number of hereditary and non hereditary diseases and conditions.

For example, "Fragile X syndrome, or Martin-Bell syndrome, is a genetic syndrome which results in a spectrum of characteristic physical, intellectual, emotional and behavioral features which range from severe to mild in manifestation. The syndrome is associated with the expansion of a single trinucleotide gene sequence (CGG) on the X chromosome, and results in a failure to express the FMR-1 protein which is required for normal neural development. There are four generally accepted forms of Fragile X syndrome which relate to the length of the repeated CGG sequence; Normal (29-31 CGG repeats) (SEQ ID NO: 6375), Premutation (55-200 CGG repeats) (SEQ ID NO: 6376), Full Mutation (more than 200 CGG repeats) (SEQ ID NO: 6377), and Intermediate or Gray Zone Alleles (40-60 repeats) (SEQ ID NO: 6378)."

Other examples include cancer, which has been associated with microsatellite instability (MSI) involving an increase or decrease in the genomic copy number of nucleic acid repeats at one or more microsatellite loci (e.g., BAT-25 and/or BAT-26). There are currently many sequencing-based assays for determining the number of nucleic acid sequence repeats at a particular locus and identifying the presence of nucleic acid insertions or deletions. However, such techniques are not useful in a high throughput multiplex analysis where the entire length of a region may not be sequenced.

In contrast, in some embodiments, aspects of the invention relate to detecting the presence of an insertion or deletion at a genomic locus without requiring the locus to be sequenced (or without requiring the entire locus to be sequenced). Aspects of the invention are particularly useful for detecting an insertion or deletion in a nucleic acid region that contains high levels of sequence repeats. The presence of sequence repeats at a genetic locus is often associated with relatively high levels of polymorphism in a population due to insertions or deletions of one or more of the sequence repeats at the locus. The polymorphisms can be associated with diseases or predisposition to diseases (e.g., certain polymorphic alleles are recessive alleles associated with a disease or condition). However, the presence of sequence repeats often complicates the analysis of a genetic locus and increases the risk of errors when using sequencing techniques to determine the precise sequence and number of repeats at that locus.

In some embodiments, aspects of the invention relate to determining the size of a genetic locus by evaluating the capture frequency of a portion of that locus suspected of containing an insertion or deletion (e.g., due to the presence of sequence repeats) using a nucleic acid capture technique (e.g., a nucleic acid sequence capture technique based on molecular inversion probe technology). According to aspects of the invention, a statistically significant difference in capture efficiency for a genetic locus of interest in different biological samples (e.g., from different subjects) is indicative of different relative lengths in those samples. It should be appreciated that the length differences may be at one or both alleles of the genetic locus. Accordingly, aspects of the invention may be used to identify polymorphisms regardless of whether biological samples being interrogated at heterozygous or homozygous for the polymorphisms. According to aspects of the invention, subjects that contain one or more loci with an insertion or deletion can be identified by analyzing capture efficiencies for nucleic acids obtained from one or more biological samples using appropriate controls (e.g., capture efficiencies for known nucleic acid sizes, capture efficiencies for other regions that are not suspected of containing an insertion or deletion in the biological sample(s), or predetermined reference capture efficiencies, or any combination thereof. However, it should be appreciated that aspects of the invention are not limited by the nature or presence of the control. In some embodiments, if a statistically significant variation in capture efficiency is detected, a subject may be identified as being at risk for a disease or condition associated with insertions or deletions at that genetic locus. In some embodiments, the subject may be analyzed in greater detail in order to determine the precise nature of the insertion or deletion and whether the subject is heterozygous or homozygous for one or more insertions or deletions. For example, gel electrophoresis of an amplification (e.g., PCR) product of the locus, or Southern blotting, or any combination thereof can be used as an orthogonal approach to verify the length of the locus. In some embodiments, a more exhaustive and detailed sequence analysis of the locus can be performed to identify the number and types of insertions and deletions. However, other techniques may be used to further analyze a locus identified as having an abnormal length according to aspects of the invention.

Accordingly, aspects of the invention relate to detecting abnormal nucleic acid lengths in genomic regions of interest. In some embodiments, the invention aims to estimate the size of genomic regions that are hard to be accessed, such as repetitive elements. However, it should be appreciated that methods of the invention do not require that the precise length be estimated. In some embodiments, it is sufficient to determine that one or more alleles with abnormal lengths are present at a locus of interest (e.g., based on the detection of abnormal capture efficiencies).

In a non-limiting example, fragile X can be used to illustrate aspects of the invention where the size of trinucleotide repeats (genotype) is linked to a symptom (phenotype). However, it should be appreciated that fragile X is a non-limiting example and similar analyses may be performed for other genetic loci (e.g., independently or simultaneously in multiplex analyses).

Use of molecular inversion probes (MIPs) has been demonstrated for detection of single nucleotide polymorphisms (Hardenbol et al. 2005 Genome Res 15:269-75) and for preparative amplification of large sets of exons (Porreca et al. 2007 Nat Methods 4:931-6, Krishnakumar et al. 2008 Proc Natl Acad Sci USA 105:9296-301). In both cases, oligonucleotide probes are designed which have ends ('targeting arms') that hybridize up-stream and down-stream of the locus that is to be amplified. In some embodiments, aspects of the invention are based on the recognition that the effect of length on probe capturing efficiency can be used in the context of an assay (e.g., a high throughput and/or multiplex assay) to allow the length of sequences to be determined without requiring sequencing of the entire region being evaluated. This is particularly useful for repeat regions that are prone to changes in size. As illustrated in FIG. **8**, which is reproduced from Deng et al., Nature Biotech. 27:353-60, (see Supplemental FIG. **1**G of Deng et al.) illustrates that shorter sequences are captured with higher efficiency that longer sequences using MIPs. The statistical package Rand its effects module were used for this analysis. A linear model was used, and each individual factor was assumed to be independent. The dashed lines represent a 95% confidence interval. Shorter target sequences were captured with higher efficiency than long target sequences (p<2×10-16). However, the use of this differential capture efficiency for systematic sequence length analysis was not previously recognized.

In some embodiments, following probe hybridization, polymerase fill-in and ligation reactions are performed to convert the hybridized probe to a covalently-closed, circular molecule containing the desired target. PCR or rolling circle amplification plus exonuclease digestion of non-circularized material is performed to isolate and amplify the circular targets from the starting nucleic acid pool. Since one of the main benefits of the method is the potential for a high degree of multiplexing, generally thousands of targets are captured in a single reaction containing thousands of probes.

According to aspects of the invention, repetitive regions are surrounded by non-repetitive unique sequences, which can be used to amplify the repeat-containing regions using, for example, PCR or padlock (MIP)-based method.

In addition to the repetitive regions, a probe (e.g., a MIP or padlock probe) can be designed to include at least a sequence that is sufficient to be uniquely identified in the genome (or target pool). After the probe is circularized and amplified, the amplicon can be end-sequenced so that the unique sequence can be identified and served as the "representative" of the repetitive region as illustrated in FIG. **9**. FIG. **9** illustrates a non-limiting scheme of padlock (MIP) capture of a region that includes both repetitive regions (thick wavy line) and the adjacent unique sequence (thick strait line). The regions of the probe are indicated with the targeting arms shown as regions "1" and "3." An intervening region that may be, or include, a sequencing primer binding site is shown as "2." After the padlock is circularized and amplified, it can be end-sequenced to obtain the sequence of the unique sequence, which represents the repetitive region of interest. Although capturing efficiency is overall negatively correlated with target length, different probe sequences may have unique features. Therefore, multiple probes could be designed and tested so that an optimal one is chosen to be sensitive enough to differentiate repetitive sizes of roughly 0-150 bp, 150-600 bp, and beyond, which represent normal, premutation and full mutation of fragile X syndrome, respectively. However, it should be appreciated that other probe sizes and sequences can be designed, and optionally optimized, to distinguish a range of repeat region size differences (e.g., length differences of about 3-30 bases, about 30-60 bases, about 60-90 bases, about 90-120 bases, about 120-150 bases, about 150-300 bases, about 300-600 bases, about 600-900 bases, or any intermediate or longer length difference). It should be appreciated that a length difference may be an increase in size or a decrease in size.

In some embodiments, an initial determination of an unexpected capture frequency is indicative of the presence of size difference. In some embodiments, an increase in capture frequency is indicative of a deletion. In some embodiments, a decrease in capture frequency is indicative of an insertion. However, it should be appreciated that depending on specific sequence parameters and the relative sizes of the capture probes, the target region, and the deletions or insertions, a change in capture frequency can be associated with either an increase or decrease in target region length. In some embodiments, the precise nature of the change can be determined using one or more additional techniques as described herein.

Accordingly, in some aspects a MIP probe includes a linear nucleic acid strand that contains two hybridization sequences or targeting arms, one at each end of the linear probe, wherein each of the hybridization sequences is complementary to a separate sequence on a the same strand of a target nucleic acid, and wherein these sequences on the target nucleic acid flank the two ends of the target nucleic acid sequence of interest. It should be appreciated that upon hybridization, the two ends of the probe are inverted with respect to each other in the sense that both 5' and 3' ends of the probe hybridize to the same strand to separate regions flanking the target region (as illustrated in FIG. **9** for example).

In some embodiments, the hybridization sequences are between about 10-100 nucleotides long, for example between about 10-30, about 30-60, about 60-90, or about 20, about 30, about 40, or about 50 nucleotides long. However, other lengths may be used depending on the application. In some embodiments, the hybridization Tins of both targeting arms of a probe are designed or selected to be similar. In some embodiments, the hybridization Tms of the targeting arms of a plurality of probes designed to capture different target regions are selected or designed to be similar so that they can be used together in a multiplex reaction. Accordingly, a typical size of a MIP probe prior to fill-in is about 60-80 nucleotides long. However, other sizes can be used depending on the sizes of the targeting arms and any other sequences (e.g., primer binding or tag sequences) that are present in the MIP probe. In some embodiments, MIP probes are designed to avoid sequence-dependent secondary structures. In some embodiments, MIP probes are designed such that the targeting arms do not overlap with known polymorphic regions. In some embodiments, targeting arms that can be used for capturing the repeat region of the Fragile X locus can have the following sequences or complementary to these sequences depending on the strand that is captured.

```
left:   CTCCGTTTCGGTTTCACTTC  (SEQ ID NO: 181)

right:  ATCTTCTCTTCAGCCCTGCT  (SEQ ID NO: 182)
```

The typical captured size using these targeting arms is about 100 nucleotides in length (e.g., about 30 repeats of a tri-nucleotide repeat).

In some embodiments, the number of reads obtained for the "representative" of the repetitive region is not informative to estimate the target length because it is dependent on the total number of reads obtained. To overcome this, it is useful to include one or more probes that target other "control" regions where no or minimal polymorphism exists among populations. Because of the systematic consistency of capturing efficiency (see, e.g., FIG. **9**), the ratio of reads obtained for the repetitive "representative" to reads obtained for the control region(s) will be tuned using DNA with defined numbers of repeats. Ultimately, the ratio can serve as a measure of the repeat length as illustrated in FIG. **10**. FIG. **10** illustrates a non-limiting hypothetical relationship between target gap size and the relative number of reads of the repetitive region, which is measured by the ratio of the repeat "representative" reads vs. the "control" region reads. The unit of y-axis is arbitrary.

In some embodiments, to better tell targets with similar size range apart, the whole repetitive region can be sequenced by making a shotgun library (e.g., by making a shotgun library from a captured sequence, for example a sequence captured using a MIP probe). The longer the repeat is, the more short reads of repeats will be obtained. Therefore, the target length will contribute twice to the relative number of "repetitive" reads, which will gain better resolution of differentiating targets. In some embodiments, the expectation is that the number of reads from any given repeat will be a direct function of the number of repeats present. However, in some embodiments, a Poisson sampling-induced spread may need to be considered and in some embodiments may be sufficiently large to limit the resolution.

When a precise measurement of the length of both alleles from a diploid sample is desired, further manipulations may be required. This is because the capture efficiency measured will actually be the average efficiency of the two alleles. To effectively achieve separate measurements for each allele, barcodes (e.g., sequence tags) can be used that allow the efficiency of individual capture events (from individual genomic loci) to be followed. FIG. **11**A-C shows the approach. For a given locus, MIPs are synthesized to contain one of a large number of differentiator tags in their backbone such that the probability of any two MIPs in a reaction having the same differentiator tag sequence is low. MIP capture is performed on the sample; the reaction will be biased for shorter target lengths, and therefore the reaction product will be comprised of more 'short' circles than 'long' circles. Each circle should bear a unique differentiator tag sequence. Then, linear RCA (lRCA) is performed on the circles. In the lRCA reaction, circles are converted into long, linear concatemers of themselves. The lRCA reaction for a given circle stops when the concatemer has reached a 'fixed' length (based on the processivity/error rate of the polymerase). Concatemers derived from smaller circles will therefore contain more copies of the differentiator tag, and concatemers derived from larger circles will contain fewer copies of the differentiator tag. The number of each differentiator tag sequence is counted, for example, by next-generation sequencing. When number of occurrences is plotted against differentiator tag ID, the data will naturally cluster into two groups reflecting the lengths of the two alleles in the diploid sample. The allele lengths can therefore be read directly off this graph, after absolute length calibration using known standards. In some embodiments, a sequencing technique (e.g., a next-generation sequencing technique) is used to sequence part of one or more captured targets (e.g., or amplicons thereof) and the sequences are used to count the number of different barcodes that are present. Accordingly, in some embodiments, aspects of the invention relate to a highly-multiplexed qPCR reaction.

Other non-limiting examples of loci at which insertions or deletions or repeat sequences may be associated with a disease or condition are provided in Tables 3 and 4. It should be appreciated that the presence of an abnormal length at any one or more of these loci may be evaluated according to aspects of the invention. In some embodiments, two or more of these loci or other loci may be evaluated in a single multiplex reaction using different probes designed to hybridize under the same reaction conditions to different target nucleic acid in a biological sample.

TABLE 3

Polyglutamine (PolyQ) Diseases

Type	Gene	Normal/ wildtype	Pathogenic
DRPLA (Dentatorubropallidoluysian atrophy)	ATN1 or DRPLA	6-35	49-88
HD (Huntington's disease)	HTT (Huntingtin)	10-35	35+
SBMA (Spinobulbar muscular atrophy or Kennedy disease)	Androgen receptor on the X chromosome.	9-36	38-62
SCA1 (Spinocerebellar ataxia Type 1)	ATXN1	6-35	49-88
SCA2 (Spinocerebellar ataxia Type 2)	ATXN2	14-32	33-77
SCA3 (Spinocerebellar ataxia Type 3 or Machado-Joseph disease)	ATXN3	12-40	55-86
SCA6 (Spinocerebellar ataxia Type 6)	CACNA1A	4-18	21-30
SCA7 (Spinocerebellar ataxia Type 7)	ATXN7	7-17	38-120
SCA17 (Spinocerebellar ataxia Type 17)	TBP	25-42	47-63

TABLE 4

Non-Polyglutamine Diseases

Type	Gene	Codon	Normal/ wildtype	Pathogenic
FRAXA (Fragile X syndrome)	FMR1, on the X-chromosome	CGG	6-53	230+

TABLE 4-continued

Non-Polyglutamine Diseases				
Type	Gene	Codon	Normal/ wildtype	Pathogenic
FXTAS (Fragile X-associated tremor/ataxia syndrome)	FMR1, on the X-chromosome	CGG	6-53	55-200
FRAXE (Fragile XE mental retardation)	AFF2 or FMR2, on the X-chromosome	GCC	6-35	200+
FRDA (Friedreich's ataxia)	FXN or X25, (frataxin)	GAA	7-34	100+
DM (Myotonic dystrophy)	DMPK	CTG	5-37	50+
SCA8 (Spinocerebellar ataxia Type 8)	OSCA or SCA8	CTG	16-37	110-250
SCA12 (Spinocerebellar ataxia Type 12)	PPP2R2B or SCA12	CAG On 5' end	7-28	66-78

The following examples illustrate aspects and embodiments of the invention and are not intended to be limiting or restrictive. Many variations of the invention will become apparent to those skilled in the art upon review of this specification. The full scope of the invention should be determined by reference to the claims, along with their full scope of equivalents, and the specification, along with such variations.

4. Increasing Detection Sensitivity:

In some embodiments, aspects of the invention relate to methods for increasing the sensitivity of nucleic acid detection assays.

There are currently many genomic assays that utilize next-generation (e.g., polony-based) sequencing to generate data, including genome resequencing, RNA-seq for gene expression, bisulphite sequencing for methylation, and Immune-seq, among others. In order to make quantitative measurements (including genotype calling), these methods utilize the counts of sequencing reads of a given genomic locus as a proxy for the representation of that sequence in the original sample of nucleic acids. The majority of these techniques require a preparative step to construct a high-complexity library of DNA molecules that is representative of a sample of interest. Current assays use one of several alternative nucleic acid preparative techniques (e.g., amplification, for example PCR-based amplification; sequence-specific capture, for example, using immobilized capture probes; or target capture into a circularized probe followed by a sequence analysis step. In order to reduce errors associated with the unpredictability (stochastic nature) of nucleic acid isolation and sequence analysis techniques, current methods to involve oversampling a target nucleic acid preparation in order to increase the likelihood that all sequences that are present in the original nucleic acid sample will be represented in the final sequence data. For example, a genomic sequencing library may contain an over- or under-representation of particular sequences from a source nucleic acid sample (e.g., genome preparation) as a result of stochastic variations in the library construction process. Such variations can be particularly problematic when they result in target sequences from a genome being absent or undetectable in a sequencing library. For example, an under-representation of particular allelic sequences (e.g., heterozygotic alleles) from a genome in a sequencing library can result in an apparent homozygous representation in a sequencing library.

In contrast, aspects of the invention relate to basing a nucleic acid sequence analysis on results from two or more different nucleic acid preparatory techniques that have different systematic biases in the types of nucleic acids that they sample rather than simply oversampling the target nucleic acid. According to some embodiments, different techniques have different sequence biases that are systematic and not simply due to stochastic effects during nucleic acid capture or amplification. Accordingly, in some embodiments, the degree of oversampling required to overcome variations in nucleic acid preparation needs to be sufficient to overcome the biases. In some embodiments, the invention provides methods that reduce the need for oversampling by combining nucleic acid and/or sequence results obtained from two or more different nucleic acid preparative techniques that have different biases.

According to the invention, different techniques have different characteristic or systematic biases. For example, one technique may bias a sample analysis towards one particular allele at a genetic locus of interest, whereas a different technique would bias the sample analysis towards a different allele at the same locus. Accordingly, the same sample may be identified as being different depending on the type of technique that is used to prepare nucleic acid for sequence analysis. This effectively represents a sensitivity issue, because each technique has a different relative sensitivities for polymorphic sequences of interest.

According to aspects of the invention, the sensitivity of a nucleic acid analysis can be increased by combining the sequences from different nucleic acid preparative steps and using the combined sequence information for a diagnostic assay (e.g., for a making a call as to whether a subject is homozygous or heterozygous at a genetic locus of interest).

Currently, the ability of DNA sequencing to detect mutations is limited by the ability of the upstream sample isolation (e.g., by amplification, immobilization enrichment, circularization capture, etc.) methods to reliably isolate the locus of interest. If one wishes to make heterozygote basecalls for a diploid genome (e.g. a human sample presented for molecular diagnostic sequencing), it is important in some embodiments that the isolation method produces near- or perfectly-uniform amounts of the two alleles to be sequenced (at least sufficiently uniform to be "called" unambiguously as a heterozygote or a homozygote for a locus of interest).

Sample preparative methods may fall into three classes: 1) single- or several-target amplification (e.g., uniplex PCR, 'multiplex' PCR), 2) multi-target hybridization enrichment (e.g., Agilent SureSelect 'hybrid capture' [Gnirke et al 2009, Nature methods 27:182-9], Roche/Nimblegen 'sequence capture' [Hodges et al 2007, Nature genetics 39:1522-7], and 3) multi-target circularization selection (e.g. molecular inversion probes or padlock probes, [Porreca et al 2007, Nature methods 4:931-6, Turner et al 2009, Nature methods 6:315-6], 'selectors' [Dahl et al 2005, Nucleic acids research 33:e71]). Each of these methods can result in a pool of isolated product that does not adequately represent the input abundance distribution. For example, the two alleles at a heterozygous position can become skewed far from their input 50:50 ratio to something that results in a missed basecall during downstream sequencing. For example, if the ratio was skewed from 50:50 to 10:90, and the sample was sequenced to 10× average coverage, there is a high probability that one of the two alleles would not be observed once in the ten sequencing reads. This would reduce the sensitivity of the sequencing method by converting a heterozygous position to homozygous (where potentially the 'mutant' allele was the one not observed). In some embodiments, a skewed ratio is a particular issue that decreases the sensitivity of detecting mutations present in a heterogeneous tumor tissue. For example, if only 10% of the cells analyzed in a heterogeneous sample harbored a heterozygous mutation, the mutation would be expected to be present in 5% of sequence reads, not 50%. In this scenario, the need for robust, sensitive detection may be even more acute.

The methods disclosed herein are based, in part, on the discovery that certain classes of isolation methods have different modes of bias. The disclosure provide methods for increasing the sensitivity of the downstream sequencing by using a combination of multiple isolation methods (e.g., one or more from at least two of the classes disclosed herein) for a sample. This is particularly important in molecular diagnostics where high sensitivity is required to minimize the chances of 'missing' a disease-associated mutation. For example, given a nominal false-negative error rate of 1×10-3 for sequencing following circularization selection, and a false-negative error rate of 1×10-3 for sequencing following hybridization enrichment, one can achieve a final false-negative rate of 1×10-6 by performing both techniques on the sample (assuming failures in each method are fully independent). For a recessive disease with carrier frequency of 0.1, caused by a single fully-penetrant mutant allele, the number of missed carrier diagnoses would decrease from 1000 per million patients tested to 1 per million patients tested. Furthermore, if the testing was used in the context of prenatal carrier screening, the number of affected children born as a result of missing the carrier call in one parent would decrease from 25 per million to 25 per billion born.

Additionally, the disclosure provides combinations of preparative methods to effectively increase sequencing coverage in regions containing disease-associated alleles. Since heterozygote error rate is largely tied to both deviations from 50:50 allele representation, and in the case of next-generation DNA sequencing deviations from average abundance (such that less abundant isolated targets are more likely to be undersampled at one or both alleles), selectively increasing coverage in these regions will also selectively increase sensitivity. Furthermore, MIPs that detect presence or absence of specific known disease-associated mutations can be used to increase sensitivity selectively. In some embodiments, these MIPs would have a targeting arm whose 3'-most region is complementary to the expected mutation, and has a fill-in length of O or more bp. Thus, the MIP will form only if the mutation is present, and its presence will be detected by sequencing.

Additionally, algorithms disclosed herein may be used to determine base identity with varying levels of stringency depending on whether the given position has any known disease-associated alleles. Stringency can be reduced in such positions by decreasing the minimum number of observed mutant reads necessary to make a consensus base-call. This will effectively increase sensitivity for mutant allele detection at the cost of decreased specificity. An embodiment of the invention combines MIPs plus hybridization enrichment, plus optionally extra MIPs targeted to specific known, common disease-associated loci, e.g., to detect the presence of a polymorphism in a target nucleic acid. A non-limiting example is illustrated in FIG. **12** that illustrates a schematic using MIPs plus hybridization enrichment, plus optionally extra MIPs targeted to specific known, common disease-associated loci, e.g., to detect the presence of a polymorphism in a target nucleic acid.

FIGS. **13** and **14** illustrate different capture efficiencies for MIP-based captures. FIG. **13** shows a graph of per-target abundance with MIP capture. In this graph, bias largely drives the heterozygote error rate, since targets which are less abundant here are less likely to be covered in sufficient depth during sequencing to adequately sample both alleles. This is from Turner et al 2009, Nature methods 6:315-6. Hybridization enrichment results in a qualitatively similar abundance distribution, but the abundance of a given target is likely not correlated between the two methods. FIG. **14** shows a graph of correlation between two MIP capture reactions from Ball et al 2009, Nature biotechnology 27:361-8. Each point represents the target abundance in replicate 1 and replicate 2. Pearson correlation r=0.956. This indicates that MIP capture reproducibly biases targets to specific abundances. Hybridization enrichment is similarly correlated from one capture to the next.

According to aspects of the invention, such biases can be detected or overcome by systematically combining different capture and/or analytical techniques in an assay that interrogates a plurality of loci in a plurality of subject samples.

Accordingly, it should be appreciated that in any of the embodiments described herein (e.g., tiling/staggering, tagging, size-detection, sensitivity enhancing algorithms, or any combination thereof), aspects of the invention involve preparing genomic nucleic acid and/or contacting them with one or more different probes (e.g., capture probes, hybridization probes, MIPs, others etc.). In some embodiments, the amount of genomic nucleic acid used per subject ranges from 1 ng to 10 micrograms (e.g., 500 ng to 5 micrograms). However, higher or lower amounts (e.g., less than 1 ng, more than 10 micrograms, 10-50 micrograms, 50-100 micrograms or more) may be used. In some embodiments, for each locus of interest, the amount of probe used per assay may be optimized for a particular application. In some embodiments, the ratio (molar ratio, for example measured as a concentration ratio) of probe to genome equivalent (e.g., haploid or diploid genome equivalent, for example for each allele or for both alleles of a nucleic acid target or locus of interest) ranges from 1/100, 1/10, 1/1, 10/1, 100/1, 1000/1. However, lower, higher, or intermediate ratios may be used.

In some embodiments, the amount of target nucleic acid and probe used for each reaction is normalized to avoid any observed differences being caused by differences in concentrations or ratios. In some embodiments, in order to normalize genomic DNA and probe, the genomic DNA concentration is read using a standard spectrophotometer or by fluorescence (e.g., using a fluorescent intercalating dye). The probe concentration may be determined experimentally or using information specified by the probe manufacturer.

Similarly, once a locus has been captured (e.g., on a MIP or other probe or in another form), it may be amplified and/or sequenced in a reaction involving one or more primers. The amount of primer added for each reaction can range from 0.1 pmol to 1 nmol, 0.15 pmol to 1.5 nmol (for example around 1.5 pmol). However, other amounts (e.g., lower, higher, or intermediate amounts) may be used.

In some embodiments, it should be appreciated that one or more intervening sequences (e.g., sequence between the first and second targeting arms on a MIP capture probe), identifier or tag sequences, or other probe sequences that are not designed to hybridize to a target sequence (e.g., a genomic target sequence) should be designed to avoid excessive complementarity (to avoid cross-hybridization) to target sequences or other sequences (e.g., other genomic sequences) that may be in a biological sample. For example, these sequences may be designed have a sufficient number of mismatches with any genomic sequence (e.g., at least 5, 10, 15, or more mismatches out of 30 bases) or as having a Tm (e.g., a mismatch Tm) that is lower (e.g., at least 5, 10, 15, 20, or more degrees C. lower) than the hybridization reaction temperature.

It should be appreciated that a targeting arm as used herein may be designed to hybridize (e.g., be complementary) to either strand of a genetic locus of interest if the nucleic acid being analyzed is DNA (e.g., genomic DNA). However, in the context of MIP probes, whichever strand is selected for one targeting arm will be used for the other one. However, in the context of RNA analysis, it should be appreciated that a targeting arm should be designed to hybridize to the transcribed RNA. It also should be appreciated that MIP probes referred to herein as "capturing" a target sequence are actually capturing it by template-based synthesis rather than by capturing the actual target molecule (other than for example in the initial stage when the arms hybridize to it or in the sense that the target molecule can remain bound to the extended MIP product until it is denatured or otherwise removed).

It should be appreciated that in some embodiments a targeting arm may include a sequence that is complementary to one allele or mutation (e.g., a SNP or other polymorphism, a mutation, etc.) so that the probe will preferentially hybridize (and capture) target nucleic acids having that allele or mutation. However, in many embodiments, each targeting arm is designed to hybridize (e.g., be complementary) to a sequence that is not polymorphic in the subjects of a population that is being evaluated. This allows target sequences to be captured and/or sequenced for all alleles and then the differences between subjects (e.g., calls of heterozygous or homozygous for one or more loci) can be based on the sequence information and/or the frequency as described herein.

It should be appreciated that sequence tags (also referred to as barcodes) may be designed to be unique in that they do not appear at other positions within a probe or a family of probes and they also do not appear within the sequences being targeted. Thus they can be used to uniquely identify (e.g., by sequencing or hybridization properties) particular probes having other characteristics (e.g., for particular subjects and/or for particular loci).

It also should be appreciated that in some embodiments probes or regions of probes or other nucleic acids are described herein as comprising or including certain sequences or sequence characteristics (e.g., length, other properties, etc.). However, it should be appreciated that in some embodiments, any of the probes or regions of probes or other nucleic acids consist of those regions (e.g., arms, central regions, tags, primer sites, etc., or any combination thereof) of consist of those sequences or have sequences with characteristics that consist of one or more characteristics (e.g., length, or other properties, etc.) as described herein in the context of any of the embodiments (e.g., for tiled or staggered probes, tagged probes, length detection, sensitivity enhancing algorithms or any combination thereof).

It should be appreciated that probes, primers, and other nucleic acids designed or used herein may be synthetic, natural, or a combination thereof. Accordingly, as used herein, the term "nucleic acid" refers to multiple linked nucleotides (i.e., molecules comprising a sugar (e.g., ribose or deoxyribose) linked to an exchangeable organic base, which is either a pyrimidine (e.g., cytosine (C), thymidine (T) or uracil (U)) or a purine (e.g., adenine (A) or guanine (G)). "Nucleic acid" and "nucleic acid molecule" may be used interchangeably and refer to oligoribonucleotides as well as oligodeoxyribonucleotides. The terms shall also include polynucleosides (i.e., a polynucleotide minus a phosphate) and any other organic base containing nucleic acid. The organic bases include adenine, uracil, guanine, thymine, cytosine and inosine. Unless otherwise stated, nucleic acids may be single or double stranded. The nucleic acid may be naturally or non-naturally occurring. Nucleic acids can be obtained from natural sources, or can be synthesized using a nucleic acid synthesizer (i.e., synthetic). Harvest and isolation of nucleic acids are routinely performed in the art and suitable methods can be found in standard molecular biology textbooks. (See, for example, Maniatis' Handbook of Molecular Biology.) The nucleic acid may be DNA or RNA, such as genomic DNA, mitochondrial DNA, mRNA, cDNA, rRNA, miRNA, or a combination thereof. Non-naturally occurring nucleic acids such as bacterial artificial chromosomes (BACs) and yeast artificial chromosomes (YACs) can also be used.

The invention also contemplates the use of nucleic acid derivatives. As will be described herein, the use of certain nucleic acid derivatives may increase the stability of the nucleic acids of the invention by preventing their digestion, particularly when they are exposed to biological samples that may contain nucleases. As used herein, a nucleic acid derivative is a non-naturally occurring nucleic acid or a unit thereof. Nucleic acid derivatives may contain non-naturally occurring elements such as non-naturally occurring nucleotides and non-naturally occurring backbone linkages.

Nucleic acid derivatives may contain backbone modifications such as but not limited to phosphorothioate linkages, phosphodiester modified nucleic acids, phosphorothiolate modifications, combinations of phosphodiester and phosphorothioate nucleic acid, methylphosphonate, alkylphosphonates, phosphate esters, alkylphosphonothioates, phosphoramidates, carbamates, carbonates, phosphate triesters, acetamidates, carboxymethyl esters, methylphosphorothioate, phosphorodithioate, p-ethoxy, and combinations thereof. The backbone composition of the nucleic acids may be homogeneous or heterogeneous.

Nucleic acid derivatives may contain substitutions or modifications in the sugars and/or bases. For example, they include nucleic acids having backbone sugars which are covalently attached to low molecular weight organic groups other than a hydroxyl group at the 3' position and other than a phosphate group at the 5' position (e.g., an 2'-O-alkylated ribose group). Nucleic acid derivatives may include non-ribose sugars such as arabinose. Nucleic acid derivatives may contain substituted purines and pyrimidines such as C-5 propyne modified bases, 5-methylcytosine, 2-aminopurine, 2-amino-6-chloropurine, 2,6-diaminopurine, hypoxanthine, 2-thiouracil and pseudoisocytosine. In some embodiments, substitution(s) may include one or more substitutions/modifications in the sugars/bases, groups attached to the base, including biotin, fluorescent groups (fluorescein, cyanine, rhodamine, etc), chemically-reactive groups including carboxyl, NHS, thiol, etc., or any combination thereof.

A nucleic acid may be a peptide nucleic acid (PNA), locked nucleic acid (LNA), DNA, RNA, or co-nucleic acids of the same such as DNA-LNA co-nucleic acids. PNA are DNA analogs having their phosphate backbone replaced with 2-aminoethyl glycine residues linked to nucleotide bases through glycine amino nitrogen and methylenecarbonyl linkers. PNA can bind to both DNA and RNA targets by

Watson-Crick base pairing, and in so doing form stronger hybrids than would be possible with DNA or RNA based oligonucleotides in some cases.

PNA are synthesized from monomers connected by a peptide bond (Nielsen, P. E. et al. Peptide Nucleic Acids, Protocols and Applications, Norfolk: Horizon Scientific Press, p. 1-19 (1999)). They can be built with standard solid phase peptide synthesis technology. PNA chemistry and synthesis allows for inclusion of amino acids and polypeptide sequences in the PNA design. For example, lysine residues can be used to introduce positive charges in the PNA backbone. All chemical approaches available for the modifications of amino acid side chains are directly applicable to PNA. Several types of PNA designs exist, and these include single strand PNA (ssPNA), bisPNA and pseudocomplementary PNA (pcPNA).

The structure of PNA/DNA complex depends on the particular PNA and its sequence. ssPNA binds to single stranded DNA (ssDNA) preferably in antiparallel orientation (i.e., with the N-terminus of the ssPNA aligned with the 3' terminus of the ssDNA) and with a Watson-Crick pairing. PNA also can bind to DNA with a Hoogsteen base pairing, and thereby forms triplexes with double stranded DNA (dsDNA) (Wittung, P. et al., Biochemistry 36:7973 (1997)).

A locked nucleic acid (LNA) is a modified RNA nucleotide. An LNA form hybrids with DNA, which are at least as stable as PNA/DNA hybrids (Braasch, D. A. et al., Chem & Biol. 8(1):1-7 (2001)). Therefore, LNA can be used just as PNA molecules would be. LNA binding efficiency can be increased in some embodiments by adding positive charges to it. LNAs have been reported to have increased binding affinity inherently.

Commercial nucleic acid synthesizers and standard phosphoramidite chemistry are used to make LNAs. Therefore, production of mixed LNA/DNA sequences is as simple as that of mixed PNA/peptide sequences. The stabilization effect of LNA monomers is not an additive effect. The monomer influences conformation of sugar rings of neighboring deoxynucleotides shifting them to more stable configurations (Nielsen, P. E. et al. Peptide Nucleic Acids, Protocols and Applications, Norfolk: Horizon Scientific Press, p. 1-19 (1999)). Also, lesser number of LNA residues in the sequence dramatically improves accuracy of the synthesis. Most of biochemical approaches for nucleic acid conjugations are applicable to LNA/DNA constructs. These and other aspects of the invention are illustrated by the following non-limiting examples.

EXAMPLES

The following examples illustrate non-limiting embodiments of the invention.

Example 1

Design a Set of Capture Probes for a Human Target Exon

All targets are captured as a set of partially-overlapping subtargets. For example, in the tiling approach, a 200 bp target exon might be captured as a set of 12 subtargets, each 60 bp in length (FIG. **1**). Each subtarget is chosen such that it partially overlaps two or three other targets.

In some embodiments, all probes are composed of three regions: 1) a 20 bp 'targeting arm' comprised of sequence which hybridizes immediately upstream from the sub-target, 2) a 30 bp 'constant region' comprised of sequence used as a pair of amplification priming sites, and

3) a second 20 bp 'targeting arm' comprised of sequence which hybridizes immediately downstream from the sub-target. Targeting arm sequences will be different for each capture probe in a set, while constant region sequence will be the same for all probes in the set, allowing all captured targets to be amplified with a single set of primers. Targeting arm sequences should be designed such that any given pair of 20 bp sequences is unique in the target genome (to prevent spurious capture of undesired sites). Additionally, melting temperatures should be matched for all probes in the set such that hybridization efficiency is uniform for all probes at a constant temperature (e.g., 60° C.). Targeting arm sequences should be computationally screened to ensure they do not form strong secondary structure that would impair their ability to basepair with the genomic target.

Hybridize Capture Probes to Human Genomic Sample

Assemble hybridization reaction:

ul capture probe mix (~2.5 pmol)

ul 10× Ampligase buffer (Epicentre)

6.0 ul 500 ng/ul human genomic DNA (–16.7 fmol) 11 ul dH2O

In a thermal cycler, heat reaction to 95° C. for 5 min to denature genomic DNA, then cool to 60° C. Allow to incubate at 60° C. for 40 hours.

Convert Hybridized Probes into Covalently-Closed Circular Products Containing Subtargets Prepare fill-in/ligation reaction mixture:

0.25 ul 2 mM dNTP mix (Invitrogen)

2.5 ul 10× Ampligase buffer (Epicentre)

5.0 ul 5 U/ul Taq Stoffel fragment (Applied Biosystems)

12.5 ul 5 U/ul Ampligase (Epicentre)

4.75 ul dH2O

Add 1.0 ul of this mix to the hybridized probe reaction, and incubate at 60° C. for 10 hours.

Purify Circularized Probe/Subtarget Products from Un-Reacted Probes and Genomic DNA

Prepare exonuclease reaction mixture:

21 ul fill-in/ligation reaction product

ul 10× exonuclease I buffer (New England Biolabs)

2.0 ul 20 U/ul exonuclease I (New England Biolabs)

2.0 ul 100 U/ul exonuclease III (New England Biolabs)

Incubate at 37° C. for 60 min, then heat-inactivate by incubating at 80° C. for 15 min.

Immediately cool to 4 C for storage.

Amplify Circular Material by PCR Using Primers Specific to the 'Constant Region' of the Probes

Prepare PCR mixture:

5.0 u 10× Accuprime reaction buffer (Invitrogen) (SEQ ID NO: 183)

1.5 ul 10 uM CP-2-FA (5'-GCACGATCCGACGGTAGTGT-3') (SEQ ID NO: 184)

1.5 ul 10 uM CP-2-RA (5'-CCGTAATCGGGAAGCTGAAG-3')

0.4 ul 25 mM dNTP mix (Invitrogen)

2.0 ul heat-inactivated exonuclease reaction mix

1.5 ul 10× SybrGreen (Invitrogen)

0.4 ul 2.5 U/ul Accuprime Pfx polymerase (Invitrogen)

37.7 ul dH2O

Thermal cycle in real-time thermal cycler according to the following protocol, but stop cycling before amplification yield plateaus (generally 8-12 cycles):

95C for 5 min 95C for 30 sec 58C for 60 sec

72C for 60 sec

go to 2, N more times

Prepare a Shotgun Next-Generation Sequencing Library for Analysis

Purify desired amplicon population from non-specific amplification products by gel extraction.

Concatemerized amplicons into high-molecular weight products suitable for shearing Mechanically shear, using either a nebulizer, BioRuptor, Hydroshear, Covaris, or similar instrument. DNA should be sheared into fragments several hundred basepairs in length.

Ligate adapters required for amplification by the sequencing platform used. If necessary, purify ligated product from unligated product and adapters.

Example 2

Use of Differentiator Tag Sequences to Detect and Correct Bias in a MIP-Capture Reaction of a Set of Exon Targets

The first step in performing the detection/correction is to determine how many differentiator tag sequences are necessary for the given sample. In this example, 1000 genomic targets corresponding to 1000 exons were captured. Since the differentiator tag sequence is part of the probe, it will measure/report biases that occur from the earliest protocol steps. Also, being located in the backbone, the differentiator tag sequence can easily be sequenced from a separate priming site, and therefore not impact the total achievable read-length for the target sequence. MIP probes are synthesized using standard column-based oligonucleotide synthesis by any number of vendors (e.g. IDT), and differentiator tag sequences are introduced as 'degenerate' positions in the backbone. Each degenerate position increases the total number of differentiator tag sequences synthesized by a factor of 4, so a 10 nt degenerate region implies a differentiator tag sequence complexity of ~1e6 species.

Hybridize Capture Probes to Human Genomic Sample

Assemble hybridization reaction:

1.0 ul capture probe mix (–2.5 pmol)

2.0 ul 10× Ampligase buffer (Epicentre)

6.0 ul 500 ng/ul human genomic DNA (~16.7 fmol) 11 ul dH2O

In a thermal cycler, heat reaction to 95° C. for 5 min to denature genomic DNA, then cool to 60° C. Allow to incubate at 60° C. for 40 hours.

Convert Hybridized Probes into Covalently-Closed Circular Products Containing Subtargets Prepare fill-in/ligation reaction mixture:

0.25 ul 2 mM dNTP mix (Invitrogen)

2.5 ul 10× Ampligase buffer (Epicentre)

5.0 ul 5 U/ul Taq Stoffel fragment (Applied Biosystems)

12.5 ul 5 U/ul Ampligase (Epicentre)

4.75 ul dH2O

Add 1.0 ul of this mix to the hybridized probe reaction, and incubate at 60° C. for 10 hours.

Purify Circularized Probe/Subtarget Products from Un-Reacted Probes and Genomic DNA

Prepare exonuclease reaction mixture:

21 ul fill-in/ligation reaction product

2.0 ul 10× exonuclease I buffer (New England Biolabs)

2.0 ul 20 U/ul exonuclease I (New England Biolabs)

2.0 ul 100 U/ul exonuclease III (New England Biolabs)

Incubate at 37° C. for 60 min, then heat-inactivate by incubating at 80° C. for 15 min. Immediately cool to 4 C for storage.

Amplify Circular Material by PCR Using Primers Specific to the 'Constant Region' of the Probes

Prepare PCR mixture:

5.0 ul 10× Accuprime reaction buffer (Invitrogen)

```
                                    (SEQ ID NO: 183)
1.5 ul 10 uM CP-2-FA   (5'-GCACGATCCGACGGTAGTGT-3')

                                    (SEQ ID NO: 184)
1.5 ul 10 uM CP-2-RA   (5'-CCGTAATCGGGAAGCTGAAG-3')
```

0.4 ul 25 mM dNTP mix (Invitrogen)

2.0 ul heat-inactivated exonuclease reaction mix

1.5 ul 10× SybrGreen (Invitrogen)

0.4 ul 2.5 U/ul Accuprime Pfx polymerase (Invitrogen)

37.7 ul dH2O

Thermal cycle in real-time thermal cycler according to the following protocol, but stop cycling before amplification yield plateaus (generally 8-12 cycles):

95C for 5 min 95C for 30 sec 58C for 60 sec 72C for 60 sec

go to 2, N more times

Prepare a shotgun next-generation sequencing library for analysis

Purify desired amplicon population from non-specific amplification products by gel extraction.

Concatemerized amplicons into high-molecular weight products suitable for shearing

Mechanically shear, using either a nebulizer, BioRuptor, Hydroshear, Covaris, or similar instrument. DNA should be sheared into fragments several hundred basepairs in length.

Ligate adapters required for amplification by the sequencing platform used. If necessary, purify ligated product from unligated product and adapters.

Perform Sequencing of Library According to Manufacturer's Directions (e.g. Illumina, ABI, etc), Reading Both the Target Sequence and the Differentiator Tag Sequence.

Analyze Data by Correcting for any Biases Detected by Quantitation of Differentiator Tag Sequence Abundance.

Construct a table of target:differentiator tag abundances from the read data, e.g.:

Target ID	Differentiator tag sequence ID	Count
1	3547	1
2	4762	1
1	9637	1
1	1078	5
3	4762	1
1	2984	1

All 'count' entries should be '1', since any particular target:differentiator tag mapping will not occur more than once by chance, and therefore will only be observed if bias was present somewhere in the sample preparation process. For any target:differentiator tag combination observed more than once, all such reads are 'collapsed' into a single read before consensus basecalls are determined. This will cancel the effect of bias on consensus basecall accuracy. FIG. **5** depicts a method for making diploid genotype calls in which repeat target:differentiator tag combination are collapsed.

Example 3

Differentiator Tag Sequence Design for MIP Capture Reactions

For a set of targets, the number of differentiator tag sequences necessary to be confident (within some statistical bounds) that a certain differentiator tag sequence will not be observed more than once by chance in combination with a certain target sequence was determined. The total number of unique differentiator tag sequences for a certain differentiator tag sequence length is determined as $4^{(\textit{Length in nucleotides of the differentiator tag sequence})}$. For a molecular inversion probe capture reaction that uses MIP probes having differentiator tag sequences, the probability of performing the capture reaction and capturing one or more copies of a target sequence having the same differentiator tag sequence is calculated as:

$p=1-[N!/(N-M)\,!]/[NM]$, wherein N is the total number of possible unique differentiator tag sequences and M is the number of target sequence copies in the capture reaction. Thus, by varying the differentiator tag sequence length it is possible to perform a MIP capture reaction in which the probability of capturing one or more copies of a target sequence having the same differentiator tag sequence is set at a predetermined probability value.

For example, for a differentiator tag sequence of 15 nucleotides in length, there are 1,073,741,824 possible differentiator tag sequences. A MIP capture reaction in which MIP probes, each having a differentiator tag sequence of 15 nucleotides, are combined with 10000 target sequence copies (e.g., genome equivalents), the probability of capturing one or more copies of a target sequence having the same differentiator tag sequence is 0.05. In this example, the MIP reaction will produce very few (usually 0, but occasionally 1 or more) targets where multiple copies are tagged with the same differentiator tag sequence. FIG. **6** depicts results of a simulation for 100000 capture reactions having 15 nucleotide differentiator tag sequences and 10000 target sequences.

Example 4

Assessment of the Probability for Obtaining Enough Sequencing Reads to Make Accurate Base-Calls at Multiple Independent Loci, as a Function of Sequencing Coverage.

Monte Carlo simulations were performed to determine sequencing coverage requirements. The simulations assume 10000 genomic copies of a given locus (target) half mom alleles and half dad alleles. The simulations further assume 1% efficiency of capture for the MIP reaction. The simulation samples from a capture mix 100 times without replacement to create a set of 100 capture products. The simulation then samples from the set of 100 capture products with replacement (assuming unbiased amplification) to generate 'reads' from either mom or dad. The number of reads sampled depends on the coverage. The number of independent reads from both mom and dad necessary to make a high-quality base-call (assumed to be 10 or 20 reads) were then determined. The process was repeated 1000 times for each coverage level, and the fraction of times that enough reads from both parents were successfully obtained was determined. This fraction was raised to the power 1000, assuming we have 1000 independent loci that must obtain successful base-calls, plotted (See FIG. **7**). Result show that roughly 50× coverage is required to capture each allele >=10× with >0.95 probability.

Example 5

MIP Capture of 'Target' Locus and 'Control' Loci

In some embodiments, to accurately quantify the efficiency of target locus capture, at least three sets of control loci are captured in parallel that have a priori been shown to serve as proxies for various lengths of target locus. For example, if the target locus is expected to have a length between 50 and 1000 bp, then sets of control loci having lengths of 50, 250, and 1000 bp could be captured (e.g. 20 loci per set should provide adequate protection from outliers), and their abundance digitally measured by sequencing. These loci should be chosen such that minimal variation in efficiency between samples and on multiple runs of the same sample is observed (and are therefore 'efficiency invariant'). These will serve as 'reference' points that define the shape of the curve of abundance-vs-length. Determining the length of the target is then simply a matter of 'reading' the length from the appropriate point on the calibration curve.

In some embodiments, the statistical confidence one has in the estimate of target length from this method is driven largely by three factors: 1) reproducibility/variation of the abundance data used to generate the calibration curve; 2) goodness of fit of the regression to the 'control' datapoints; 3) reproducibility of abundance data for the target locus being measured. Statistical bounds on 1) and 2) will be known in advance, having been measured during development of the assay. Additionally, statistical bounds on 3) will be known in general in advance, since assay development should include adequate population sampling and measure of technical reproducibility. Standard statistical methods should be used to combine these three measures into a single P value for any given experimental measure of target abundance.

In some embodiments, given the set of calibration observations, and a linear regression fit to that data, the regression can be used to predict the length value for n observations of the target locus whose length is unknown. First, choose an acceptable range for the confidence interval of the length estimate. For example, in the case of distinguishing "normal" (87-93 bp) from "premutation" (165-600 bp) potential cases of Fragile X, the goal is to measure length to sufficient precision to distinguish 93 bp from 165 bp. The predicted response value, computed when n observations is substituted into the equation for the regressed line, will have arbitrary precision. However, if for example a 95% confidence level is desired, that 95% confidence interval must be sufficiently short that it does not overlap both the "normal" and "premutation" length ranges. Continuing the example, if one calculates a length of 190 from n=400 MIP observations, and based on the regression from calibration data, the 95% confidence interval is 190+/−20 bp, one can conclude the sample represents a "premutation" length with 95% certainty. Conversely, if the calibration data were less robust, error estimates of the regression would be higher, leading to larger confidence intervals on the predicted response value. In some embodiments, if the 95% CI were calculated as 190+/−100 bp from n=400, one could not determine whether the predicted response value corresponds to a "normal" or "premutation" length.

In some embodiments, the confidence interval for a predicted response is calculated as:

The estimate for the response $\hat{y}$ is identical to the estimate for the mean of the response: {tilde over (y)}$=b_0+b_1x^*$. The confidence interval for the predicted value is given by

$\hat{y}\pm t^{*}s_{\hat{y}}$, where $\hat{y}$ is the fitted value corresponding to x^{*}. The value t^{*} is the upper $(1-C)/2$ critical value for the $t(n-2)$ distribution.

In some embodiments, a technique for analyzing a locus of interest can involve the following steps.

Convert Hybridized Probes into Covalently-Closed Circular Products Containing Subtargets

Prepare fill-in/ligation reaction mixture:

0.25 ul 2 mM dNTP mix (Invitrogen)

2.5 ul 10× Ampligase buffer (Epicentre)

5.0 ul 5 U/ul Taq Stoffel fragment (Applied Biosystems)

12.5 ul 5 U/ul Ampligase (Epicentre)

4.75 ul dH2O

Add 1.0 ul of this mix to the hybridized probe reaction, and incubate at 60° C. for 10 hours.

Purify Circularized Probe/Subtarget Products from Un-Reacted Probes and Genomic DNA

Prepare exonuclease reaction mixture:

21 ul fill-in/ligation reaction product

2.0 ul 10× exonuclease I buffer (New England Biolabs)

2.0 ul 20 U/ul exonuclease I (New England Biolabs)

2.0 ul 100 U/uI exonuclease III (New England Biolabs)

Incubate at 37° C. for 60 min, then heat-inactivate by incubating at 80° C. for 15 min. Immediately cool to 4 C for storage.

Amplify Circular Material by PCR Using Primers Specific to the 'Constant Region' of the Probes

Prepare PCR mixture:

5.0 ul 10× Accuprime reaction buffer (Invitrogen)

1.5 ul 10 uM CP-2-FA-Ilmn (platform-specific amplification sequence plus 'circle constant region'-specific sequence)

1.5 ul 10 uM CP-2-RA-Ilmn (platform-specific amplification sequence plus 'circle constant region'-specific sequence)

0.4 ul 25 mM dNTP mix (Invitrogen)

2.0 ul heat-inactivated exonuclease reaction mix

1.5 ul 10× SybrGreen (Invitrogen)

0.4 ul 2.5 U/ul Accuprime Pfx polymerase (Invitrogen)

37.7 ul dH2O

Thermal cycle in real-time thermal cycler according to the following protocol, but stop cycling before amplification yield plateaus (generally 8-12 cycles):

95C for 5 min 95C for 30 sec 58C for 60 sec 72C for 60 sec

go to 2, N more times

Perform Sequencing (e.g., Next-Generation Sequencing) on Sample for Digital Quantitation According to Manufacturer's Instructions (e.g., Illumina, Abi)

Example 6

MIP-Capture Reaction of a Set of Exon Target Nucleic Acids

MIP probes are synthesized using standard column-based oligonucleotide synthesis by any number of vendors (e.g. IDT).

Hybridize Capture Probes to Human Genomic Sample Assemble hybridization reaction:

1.0 ul capture probe mix (~2.5 pmol)

2.0 ul 10× Ampligase buffer (Epicentre)

6.0 ul 500 ng/ul human genomic DNA (~16.7 fmol) 11 ul dH2O

In a thermal cycler, heat reaction to 95° C. for 5 min to denature genomic DNA, then cool to 60° C. Allow to incubate at 60° C. for 40 hours.

Convert Hybridized Probes into Covalently-Closed Circular Products Containing Target Nucleic Acids

Prepare fill-in/ligation reaction mixture:

0.25 ul 2 mM dNTP mix (Invitrogen)

2.5 ul 10× Ampligase buffer (Epicentre)

5.0 ul 5 U/ul Taq Stoffel fragment (Applied Biosystems)

12.5 ul 5 U/ul Ampligase (Epicentre)

4.75 ul dH2O

Add 1.0 ul of this mix to the hybridized probe reaction, and incubate at 60° C. for 10 hours.

Purify circularized probe/target nucleic acid products from un-reacted probes and genomic DNA

Prepare exonuclease reaction mixture:

21 ul fill-in/ligation reaction product

2.0 ul 10× exonuclease I buffer (New England Biolabs)

2.0 ul 20 U/ul exonuclease I (New England Biolabs)

2.0 ul 100 U/ul exonuclease III (New England Biolabs)

Incubate at 37° C. for 60 min, then heat-inactivate by incubating at 80° C. for 15 min. Immediately cool to 4° C. for storage.

Amplify Circular Material by PCR Using Primers Specific to the 'Constant Region' of the Probes

Prepare PCR mixture:

5.0 ul 10× Accuprime reaction buffer (Invitrogen)

```
                                        (SEQ ID NO: 183)
1.5 ul 10 uM CP-2-FA    (5'-GCACGATCCGACGGTAGTGT-3')

                                        (SEQ ID NO: 184)
1.5 ul 10 uM CP-2-RA    (5'-CCGTAATCGGGAAGCTGAAG-3')
```

0.4 ul 25 mM dNTP mix (Invitrogen)

2.0 ul heat-inactivated exonuclease reaction mix

1.5 ul 10× SybrGreen (Invitrogen)

0.4 ul 2.5 U/ul Accuprime Pfx polymerase (Invitrogen)

37.7 ul dH2O

Thermal cycle in real-time thermal cycler according to the following protocol, but stop cycling before amplification yield plateaus (generally 8-12 cycles):

95° C. for 5 min

95° C. for 30 sec

58° C. for 60 sec

72° C. for 60 sec

go to 2, N more times

Prepare a Shotgun Next-Generation Sequencing Library for Analysis

Purify desired amplicon population from non-specific amplification products by gel extraction.

Concatemerized amplicons into high-molecular weight products suitable for shearing Mechanically shear, using either a nebulizer, BioRuptor, Hydroshear, Covaris, or similar instrument. DNA should be sheared into fragments several hundred basepairs in length.

Ligate adapters required for amplification by the sequencing platform used. If necessary, purify ligated product from unligated product and adapters.

Perform Sequencing of Library According to Manufacturer's Directions (e.g. Illumina, ABI, etc), Reading the Target Sequence to Determine Abundance of the Target Nucleic Acid.

Example 7

Use of MIPs, Hybridization, and Mutation-Detection MIPs to Genotype a Set of 1000 Targets MIPs, hybridization, and mutation-detection MIPs are used to genotype a set of 1000 targets. The protocol permits detection of any of 50 specific known point mutations First, separate MIP, hybridization, and mutation-detection MIP reactions are performed on a biological sample. A MIP capture reaction is performed essentially as described in Turner et al 2009, Nature methods 6:315-6. A set of MIPs is designed such to that each probe in the set flanks one of the 1000 targets. Separately, a hybridization enrichment reaction is performed using the Agilent SureSelect procedure. Prior to selection, the genomic DNA to be enriched is converted into a shotgun sequencing library using Illumina's 'Fragment Library' kit and protocol. Agilent's web interface is used to design a set of probes which will hybridize to the target nucleic acids. Separately, a set of probes are designed (mutation-detection MIPs) which will form MIPs only if mutations (e.g., specific polymorphisms) are present. Each mutation-detection MIP has a 3'-most base identity that is specific for a single known mutation. A reaction with this set of mutation-detection MIPs is performed to selectively detect the presence of any mutant alleles.

Once all three reactions have been performed, the two MIP reactions are combined (e.g., at potentially non-equimolar ratios to further increase sensitivity of mutation detection) into a single tube, and run as one sample on the next-generation DNA sequencing instrument. The hybridization-enriched reaction is run as a separate sample on the next-generation DNA sequencing instrument. Reads from each 'sample' are combined by a software algorithm which forms a consensus diploid genotype at each position in the target set by evaluating the total coverage at each position, the origin of each read in that total coverage, the quality score of each individual read, and the presence (or absence) of any reads derived from mutation-specific MIPs overlapping the region.

Example 8

Carrier screening is performed either pre-conception or during pregnancy to determine a couple's risk of having a child with a recessive genetic disorder. The number of individuals who could benefit from such screening is substantial, as roughly 2 million women give birth to their first child each year in the US. The disorders for which testing is recommended vary based on a number of different patient-specific factors. For instance, the American Congress of Obstetricians and Gynecologists recommends that screening for cystic fibrosis be offered to all women of reproductive age, and that testing be performed for additional disorders if indicated by family history, partner's carrier status, or ethnicity.

Today, carrier screening is typically performed using focused genotyping technologies that are designed to interrogate specific mutations within a gene of interest. However, because of cost and complexity, these tests often do not include all known disease causing mutations. In contrast, next-generation DNA sequencing (NGS) can comprehensively genotype a set of genes in a cost-efficient manner, and is therefore poised to supplant current technologies for routine, high-volume carrier screening.

For NGS to be used for carrier screening in a clinical setting, it must satisfy at least three requirements. First, analytical accuracy must be both high and well characterized within the clinically relevant genes or regions. Previous reports have demonstrated a broad range of accuracy values, and in some cases it is unclear whether these values hold within the relevant regions of the genome. In addition, accuracy for insertions and deletions is generally either substantially lower or uncharacterized, and measured to lower precision. Second, the NGS workflow employed should yield data sufficient to cover the vast majority of targeted bases at a depth sufficient to make high-quality genotype calls. It has been noted, however, that the percentage of bases callable at a given depth varies widely with both the sample preparation workflow and the total amount of sequencing 8,10. Finally, the workflow must be highly robust and reproducible, which can often be achieved through automation. However, typical NGS sample preparation workflows are not amenable to high-throughput automation because of rate-limiting mechanical shearing, reaction purifications, size selections, and kitted reagent costs (typically $50-$200 per sample).

The following is an integrated NGS workflow that meets these requirements for carrier screening. The workflow combines automated, optimized molecular inversion probe target capture with molecular barcoding to maximize the sample throughput of a next-generation DNA sequencing machine, and employs a novel read assembly-based alignment method that enables accurate identification of both substitution and insertion/deletion lesions. The workflow is applied to sequence the protein-coding regions of fifteen genes in which loss-of-function mutations cause recessive Mendelian disorders often included as part of routine carrier screening, and demonstrate through realistic simulation and comparison to Sanger sequencing data that our approach achieves high accuracies.

METHODS AND MATERIALS

Molecular Inversion Probe Design

Molecular inversion probes were designed to capture the coding regions and certain well-characterized non-coding regions of 15 genes (See Table 5 below). The 5' targeting arm (ligation arm) and 3' targeting arm (extension arm) comprised a total of 40 nucleotides and were designed to flank 130 bp target regions. Probes were selected to maximize performance with respect to both capture efficiency and robustness to common polymorphisms. All possible probes targeting a genomic interval were designed and assigned score tuples consisting of: 1) presence of guanine or cytosine as the 5'-most base of the ligation arm, 2) the number of dbSNP (version 130) entries intersecting targeting arm sites, and 3) the root mean squared deviation of the arms' predicted melting temperatures from optimal values derived from empirical studies of capture efficiency.

Using these tuples, probes were ranked sequentially by 1, 2, and 3, and the probe with the highest rank was chosen. Probes were designed to 'tile' across targets with a period of 25 bp such that multiple probes with orthogonal targeting arm sequences captured every genomic position. The molecular inversion probes are provided in Appendix A. Appendix A also includes the upstream and downstream regions corresponding to each molecular inversion probe, which is shown by the start position and end position coordinates of each targeting arm relative to the target sub-region's coordinates on the Human Genome 18 (HG 18). Appendix B lists the genomic sub-regions targeted by the molecular inversion probes of Appendix A.

Table 5 shows diseases and genes the workflow is designed to interrogate, and the corresponding genes and nucleotides targeted.

TABLE 5

DISEASE	OMIM ID	GENE	NT TARGETED
Familial hyperinsulinism	256450	ABCC8	5,808
Canavan disease	271900	ASPA	1,062
Maple syrup urine disease type 1a/1b	248600	BCKDHA	1,518
		BCKDHB	1,379
Bloom syndrome	210900	BLM	4,674
Cystic fibrosis	219700	CFTR	5,444
Usher syndrome type IIIA	276902	CLRN1	856
Dihydrolipoamide dehydrogenase deficiency	248600	DLD	1,810
Fanconi anemia group C	227645	FANCC	1,957
Glycogen storage disease type 1a	232200	G6PC	1,174
Tay-Sachs disease	272800	HEXA	1,870
Familial dysautonomia	223900	IKBKAP	4,719
Mucolipidosis type IV	252650	MCOLN1	2,023
Usher syndrome type IF	602083	PCDH15	6,508
Niemann-Pick disease type A/B	257200/ 607616	SMPD1	2,056
		TOTAL	42,858

Target Capture, Barcoding, and NGS

Genomic DNA was purchased from the Coriell Cell Repositories (Camden, NJ) or isolated from whole blood by the Gentra Puregene method (Qiagen) modified to conclude with an overnight incubation at 65° C. Overnight incubation at an elevated temperature led to DNA shearing and an increased fraction of callable bases. All samples were considered “IRB Exempt” by Liberty IRB, our independent Institutional Review Board. On Tecan automation, 1.5 ug of genomic DNA was annealed with 1 ul of molecular inversion probe mix in 1× Ampligase buffer (Epicentre Biotechnologies) for 5 min at 95° C. followed by 24 hr at 54° C. 17 ul of fill-in mix (4 U Taq Stoffel fragment [Life Technologies], 10 U Ampligase [Epicentre Biotechnologies], 23.1 uM dNTP mix) was added by Tecan automation and incubated for 1 hr at 54° C. 50 U Exonuclease I and 50 U Exonuclease III (Enzymatics Inc.) were then added by Tecan automation and incubated for 1 hr at 37° C. followed by 10 min at 98° C. The capture reaction product was amplified in two separate PCR reactions designed to attach a molecular barcode and Illumina cluster amplification sequences to the ends of each molecule so as to enable sequencing from each end of the captured region. Tecan automation was used to set up the PCR, which was carried out with 3.75 ul of capture product, 15 pmol of each primer, 10 nmol dNTPs, and 1 U VeraSeq polymerase (Enzymatics, Inc) in 1× Veraseq buffer. Cycling conditions were: 98° C. 30 see, 17-22× (98° C. 10 sec, 54° C. 30 see, 72° C. 15 sec), 4° C. forever.

Following PCR, equal volumes of product from multiple samples were pooled using Tecan automation, then purified using a Qiaquick column (Qiagen). The library pool concentration was quantified on a Bioanalyzer 2100 (Agilent Technologies) and diluted to 10 nM. Single-read sequencing (85 bp for genomic tag and 15 bp for barcode/index) was performed on the Hiseq 2000 (Illumina, Inc) according to the manufacturer’s instructions. Each pool of libraries was sequenced in 7 lanes, with the 8th lane used for the manufacturer-supplied PhiX control library.

NGS Data Analysis with Alignment Only Algorithm

Raw.bcl files were converted to qseq files using bclConverter (Illumina). Fastq files were generated by ‘de-barcoding’ genomic reads using the associated barcode reads; reads for which barcodes yielded no exact match to an expected barcode, or contained one or more low-quality basecalls, were discarded. The remaining reads were aligned to hg18 on a per-sample basis using BWA version 0.5.7 for short alignments and genotype calls were made using GATK version 1.0.4168 after base quality score re-calibration, realignment (with GATK version 1.0.5083) and targeting arm removal. High-confidence genotype calls were defined as having depth >=50 and strand bias score<=0. Clinical significance of variant calls was determined by matching against a VCF-formatted database of disease-causing mutations curated from the literature, with equivalent insertion/deletion regions calculated as previously described.

NGS Data Analysis with Genotyping by Assembly-Templated Alignment Algorithm

De-barcoded fastq files were obtained as described above and partitioned by capture region (exon) using the target arm sequence as a unique key. Reads were assembled in parallel by exon using SSAKE version 3.7 with parameters “–m 30–o 15”. The resulting contigs were aligned to hg18 using BWA version 0.5.7 for long alignments with parameter “–r 1”. Short read alignment was performed as described above except that sample contigs (rather than hg18) were used as the input reference sequence. Software was developed in Java to accurately transfer coordinate and variant data (gaps) from local sample space to global reference space for every BAM-formatted alignment. Genotyping and base quality recalibration were performed on the coordinate-translated BAM files using GATK version 1.6.5.

Sanger Sequencing

PCR was carried out with the genomic DNA described in Target capture, barcoding, and NGS using a modified version of the protocol from Zimmerman et al., using PCR primers from Jones et al., except M13 tails were removed. See Zimmerman R S, Cox S, Lakdawala N K, et al. A novel custom resequencing array for dilated cardiomyopathy. Genet Med. May 2010; 12(5): 268-278; Jones S, Zhang X, Parsons D W, et al. Core signaling pathways in human pancreatic cancers revealed by global genomic analyses. Science. Sep. 2008; 321(5897):1801-1806.

Briefly, 15 ul reactions were performed with 25 ng of genomic DNA, 1U of AmpliTaq Gold (Applied Biosystems), and 10 fmol of each PCR primer in a PCR mix containing 4.8% DMSO (v/v), IM betaine, 2.5 mM magnesium chloride, 1 uM dNTPs (total), and 1× GeneAmp PCR Gold Buffer (Applied Biosystems). Cycling conditions were: 95° C. 10 min, 30×(95° C. 30 see, 60° C. 30 sec, 72° C. 30 sec), 72° C. 10 min, 8° C. forever. PCR products were sent to either Beckman Coulter Genomics or Genewiz where cleanup and chain termination bi-directional Sanger sequencing was performed on an ABI 3730xl according to standard protocols. Data was retrieved in electropherogram (abl) format.

Sanger Data Analysis and Cross-Validation to NGS

Mutation Surveyor software (“MS”, Softgenetics) version 4.0.5 was used in batch-mode with default parameters to align abl files to target reference sequence and make genotype calls. Positions where MS base calls did not match in the forward and reverse directions were removed from consideration. All high-quality NGS genotype calls within 10 bp (inclusive) of target exons were subjected to cross-validation against VCF-converted MS variant calls. This process is described in more detail below.

Calls were compared by (i) lesion type (substitution, insertion, deletion, or combination thereof), (ii) lesion pattern (sequence difference compared to the reference), and (iii) genomic position (or equivalent position for insertions and deletions). NGS calls were classified true positive (TP), discordant (non-reference) variant genotype (DVG), or false positive (FP) if they matched MS calls by (i-iii), (iii) only, or none of the above criteria, respectively. MS variant calls with no corresponding NGS variant call were classified false negative (FN). Indel calls classified as DVG were reclassified as TP because GATK 1.0.4168 does not report zygosity for such calls. All concordant reference calls were considered true negative (TN). Each discordant call (DVG, FP, and FN), along with a subset of concordant calls, was subject to expert manual review and discarded or reclassified as appropriate. False positive rate was calculated as FP/(FP+TN).

False negative rate was calculated as FN/(FN+TP). Compound heterozygous NGS calls (two different non-reference alleles) were cross-validated against Sanger data manually by aligning traces to a reference manipulated to contain one of the two variant alleles. In these cases TP genotype calls were reported as simple heterozygous by MS.

Assessment of Detectability of Clinical Mutations by Simulation

145 Coriell samples were sequenced and analyzed by Genotyping by Assembly-Templated Alignment (GATA, described above). Applications were developed in Java and Groovy to input aligned reads (BAM records) from each sample and manipulate specific data fields (base sequence and qualities) to resemble the appropriate DNA lesion pattern of a given clinically relevant mutation. To simulate heterozygous carriers input reads covering the mutation were chosen at random for sequence manipulation with an average probability of 0.5. All reads, whether manipulated or not, were output in fastq format for subsequent GATA analysis as described. This process was repeated for each of 81 mutations of clinical significance whereupon genotyped (observed) alleles were cross-referenced back to the original simulated (expected) allele. Samples for which the allele was already present were excluded from simulation (e.g. many Coriell samples in the set contained the common CFTR F508del mutation). Mutations with detection rates <100% between the expected and observed alleles were classified as undetectable by NGS.

Determining Clinical Significance of Variant Allele Calls

Each NOS-detected variant allele is annotated for functional (clinical) significance by determining its relative position within the corresponding consensus coding sequence (CCDS). For the genes under consideration here these are: PCDH15 (CCDS7248.1), SMPD1 (CCDS44531.1), ABCC8 (CCDS31437.1), HEXA (CCDS10243.1), BLM (CCDS10363.1), ASPA (CCDS11028.1), G6PC (CCDS11446.1), MCOLN1 (CCDS12180.1), BCKDHA (CCDS12581.1), CLRN1 (CCDS3153.1), BCKDHB (CCDS4994.1), DLD (CCDS5749.1), CFTR (CCDS5773.1), FANCC (CCDS35071.1), and IKBKAP (CCDS6773.1). Clinically significant (reportable) mutations include alterations to the conserved 2 basepairs flanking each exon (splice site), the native start codon, or the last codon (readthrough), as well as truncating (nonsense and frameshift) mutations. Additionally, GATK occasionally reports alternate insertion patterns with non-native bases (e.g. 'N') chosen from a minority of reads. These were classified 'indeterminate' and reportable to prompt follow-up confirmation.

Results

i. Completeness and Reproducibility

Automated target capture and molecular barcoding were performed followed by NGS on a set of 194 samples derived from immortalized cell lines (55 containing specific disease-causing mutations, and 139 chosen to represent ethnic diversity) and 59 samples derived from whole blood (as shown in Table 6 below). All exons were targeted including 10 nt of flanking intronic sequence, plus additional intronic regions known to contain disease-causing mutations in 15 genes causative of 14 recessive Mendelian diseases (Table 5) using tiling molecular inversion probes (see Methods). A total of 25,907,612,945 basepairs of de-multiplexed sequence were generated, corresponding to an average per-base coverage per sample of 2,399× (m 891×, max 4,000×). Out of the 42,858 bases targeted for capture in each sample, we made high-confidence genotype calls at an average of 97.3% (m 92.2%, max 99.8%) for cell line-derived DNA and 99.9% (m 99.8%, max 99.9%) for blood-derived DNA (See Table 5 above).

Table 6 shows the set of 94 samples derived from immortalized cell lines and 59 samples derived from whole blood.

TABLE 6

Sample ID	Sample Source	Sample Type	Sequence (raw bp)	Average Coverage	Percentage of Bases >= 50X	Reproducibility ?	Sanger Concordance ?
GM00502	Cell line	Disease	76,288,149	1,787	97.6	Yes	Yes
GM00649	Cell line	Disease	115,317,695	2,701	97.9	Yes	Yes
GM00650	Cell line	Disease	69,572,569	1,629	93.1	Yes	Yes
GM01531	Cell line	Disease	93,831,687	2,198	99.2	Yes	Yes
GM02533	Cell line	Disease	61,190,070	1,433	98.6	Yes	Yes
GM02828	Cell line	Disease	49,081,409	1,150	98.6	Yes	Yes
GM03252	Cell line	Disease	47,780,116	1,119	98.9	Yes	Yes
GM03461	Cell line	Disease	133,932,433	3,137	95.2	Yes	Yes
GM04268	Cell line	Disease	113,557,310	2,660	98.6	Yes	Yes
GM04330	Cell line	Disease	115,608,790	2,708	94.3	Yes	Yes
GM05042	Cell line	Disease	113,811,449	2,666	96.0	Yes	Yes
GM06966	Cell line	Disease	51,321,434	1,202	99.0	Yes	Yes

TABLE 6-continued

Sample ID	Sample Source	Sample Type	Sequence (raw bp)	Average Coverage	Percentage of Bases >= 50X	Reproducibility ?	Sanger Concordance ?
GM07381	Cell line	Disease	87,834,174	2,057	99.3	Yes	Yes
GM07441	Cell line	Disease	108,471,717	2,541	99.4	Yes	Yes
GM07552	Cell line	Disease	106,594,630	2,497	99.3	Yes	Yes
GM07732	Cell line	Disease	137,685,131	3,225	94.7	Yes	Yes
GM07857	Cell line	Disease	98,376,083	2,304	96.6	Yes	Yes
GM08338	Cell line	Disease	131,459,591	3,079	96.9	Yes	Yes
GM11275	Cell line	Disease	119,881,299	2,808	99.3	Yes	Yes
GM11277	Cell line	Disease	85,993,084	2,014	99.3	Yes	Yes
GM11278	Cell line	Disease	125,921,303	2,949	92.9	Yes	Yes
GM11280	Cell line	Disease	121,485,712	2,845	99.2	Yes	Yes
GM11281	Cell line	Disease	107,022,433	2,507	99.7	No	Yes
GM11282	Cell line	Disease	105,909,029	2,481	99.5	Yes	Yes
GM11283	Cell line	Disease	128,624,241	3,013	96.5	Yes	Yes
GM11284	Cell line	Disease	125,265,008	2,934	99.8	Yes	Yes
GM11285	Cell line	Disease	105,205,580	2,464	97.3	Yes	Yes
GM11287	Cell line	Disease	121,267,787	2,840	96.4	Yes	Yes
GM11288	Cell line	Disease	116,071,397	2,719	98.0	Yes	Yes
GM11370	Cell line	Disease	106,105,647	2,485	95.9	Yes	Yes
GM11468	Cell line	Disease	120,852,669	2,831	97.9	Yes	Yes
GM11472	Cell line	Disease	146,901,682	3,441	97.0	Yes	Yes
GM11496	Cell line	Disease	119,104,149	2,790	99.3	Yes	Yes
GM11497	Cell line	Disease	103,338,594	2,420	99.0	Yes	Yes
GM11723	Cell line	Disease	111,446,565	2,610	99.1	Yes	Yes
GM11859	Cell line	Disease	132,558,321	3,105	99.7	Yes	Yes
GM11860	Cell line	Disease	133,241,170	3,121	99.5	Yes	Yes
GM12444	Cell line	Disease	112,979,130	2,646	99.3	Yes	Yes
GM12585	Cell line	Disease	142,275,400	3,332	98.4	Yes	Yes
GM12785	Cell line	Disease	77,781,835	1,822	99.0	Yes	Yes
GM12960	Cell line	Disease	105,134,323	2,462	99.2	Yes	Yes
GM13205	Cell line	Disease	116,426,912	2,727	97.0	Yes	Yes
GM13423	Cell line	Disease	142,163,141	3,330	96.5	Yes	Yes
GM13591	Cell line	Disease	127,607,783	2,989	98.6	Yes	Yes
GM16193	Cell line	Disease	80,190,257	1,878	92.7	Yes	Yes
GM17023	Cell line	HuVar	114,118,598	2,673	98.9	No	Yes
GM17074	Cell line	TGP	125,212,956	2,933	98.5	No	Yes
GM17075	Cell line	TGP	114,067,841	2,672	95.1	No	Yes
GM17078	Cell line	TGP	135,956,925	3,184	97.3	No	Yes
GM17079	Cell line	TGP	109,131,650	2,556	93.8	No	Yes

TABLE 6-continued

Sample ID	Sample Source	Sample Type	Sequence (raw bp)	Average Coverage	Percentage of Bases >= 50X	Reproducibility ?	Sanger Concordance ?
GM17080	Cell line	TGP	104,079,000	2,438	98.2	No	Yes
GM17203	Cell line	HuVar	100,286,170	2,349	95.8	No	Yes
GM17207	Cell line	HuVar	133,095,165	3,117	99.7	No	Yes
GM17228	Cell line	HuVar	75,792,351	1,775	98.7	No	Yes
GM17231	Cell line	HuVar	138,157,418	3,236	97.1	No	Yes
GM17233	Cell line	HuVar	115,522,256	2,706	97.1	No	Yes
GM17242	Cell line	HuVar	114,147,392	2,673	99.3	No	Yes
GM17247	Cell line	HuVar	88,905,331	2,082	99.3	No	Yes
GM17251	Cell line	HuVar	134,029,728	3,139	97.8	No	Yes
GM17282	Cell line	HuVar	104,284,777	2,443	95.5	Yes	Yes
GM17286	Cell line	HuVar	124,885,886	2,925	98.0	No	Yes
GM17301	Cell line	HuVar	115,253,375	2,699	95.5	Yes	Yes
GM17302	Cell line	HuVar	126,663,091	2,967	95.6	Yes	Yes
GM17303	Cell line	HuVar	148,723,815	3,483	96.8	No	Yes
GM17304	Cell line	HuVar	140,507,360	3,291	95.3	No	Yes
GM17310	Cell line	HuVar	112,930,123	2,645	96.5	No	Yes
GM17315	Cell line	HuVar	146,713,295	3,436	96.0	Yes	Yes
GM17317	Cell line	HuVar	120,214,964	2,816	96.1	No	Yes
GM17318	Cell line	HuVar	131,177,753	3,072	98.4	Yes	Yes
GM17319	Cell line	HuVar	74,599,530	1,747	96.3	Yes	Yes
GM17320	Cell line	HuVar	143,908,026	3,371	98.9	Yes	Yes
GM17360	Cell line	HuVar	72,217,715	1,691	99.3	Yes	Yes
GM17361	Cell line	HuVar	138,241,789	3,238	97.5	No	Yes
GM17362	Cell line	HuVar	109,391,827	2,562	95.4	Yes	Yes
GM17363	Cell line	HuVar	136,216,563	3,190	97.4	No	Yes
GM17364	Cell line	HuVar	124,580,794	2,918	98.8	Yes	Yes
GM17365	Cell line	HuVar	145,974,763	3,419	96.8	Yes	Yes
GM17366	Cell line	HuVar	121,059,291	2,835	95.3	Yes	Yes
GM17367	Cell line	HuVar	124,286,280	2,911	97.7	Yes	Yes
GM17368	Cell line	HuVar	122,309,228	2,865	97.4	Yes	Yes
GM17369	Cell line	HuVar	151,606,788	3,551	97.2	No	Yes
GM17392	Cell line	HuVar	120,466,852	2,822	96.9	Yes	Yes
GM17393	Cell line	HuVar	129,362,199	3,030	96.1	Yes	Yes
GM17394	Cell line	HuVar	133,049,780	3,116	96.4	No	Yes
GM17395	Cell line	HuVar	145,469,089	3,407	97.0	No	Yes
GM17396	Cell line	HuVar	131,796,124	3,087	96.1	No	Yes
GM17962	Cell line	HapMap	110,772,396	2,594	92.3	Yes	Yes
GM17965	Cell line	HapMap	131,430,391	3,078	95.4	No	Yes

TABLE 6-continued

Sample ID	Sample Source	Sample Type	Sequence (raw bp)	Average Coverage	Percentage of Bases >= 50X	Reproducibility ?	Sanger Concordance ?
GM17966	Cell line	HapMap	108,405,815	2,539	92.6	No	Yes
GM17967	Cell line	HapMap	133,849,482	3,135	95.2	Yes	Yes
GM17968	Cell line	HapMap	104,839,659	2,455	96.0	Yes	Yes
GM17969	Cell line	HapMap	170,762,900	4,000	98.7	Yes	Yes
GM17970	Cell line	HapMap	129,700,472	3,038	96.5	Yes	Yes
GM17971	Cell line	HapMap	146,346,722	3,428	97.2	Yes	Yes
GM17972	Cell line	HapMap	139,495,486	3,267	96.2	Yes	Yes
GM17973	Cell line	HapMap	116,085,421	2,719	93.2	No	Yes
GM18015	Cell line	HuVar	120,955,000	2,833	95.2	No	Yes
GM18017	Cell line	HuVar	128,904,006	3,019	96.0	Yes	Yes
GM18034	Cell line	HuVar	104,807,682	2,455	97.4	Yes	Yes
GM18043	Cell line	HuVar	117,441,953	2,751	95.4	No	Yes
GM18044	Cell line	HuVar	149,341,518	3,498	96.8	No	Yes
GM18067	Cell line	HuVar	90,615,125	2,122	94.3	Yes	Yes
GM18073	Cell line	HuVar	120,359,154	2,819	94.0	No	Yes
GM18075	Cell line	HuVar	130,655,292	3,060	96.1	No	Yes
GM18084	Cell line	HuVar	127,693,612	2,991	97.0	No	Yes
GM18087	Cell line	HuVar	116,883,425	2,738	95.3	Yes	Yes
GM18089	Cell line	HuVar	113,522,775	2,659	93.8	No	Yes
GM18090	Cell line	HuVar	139,175,351	3,260	95.6	Yes	Yes
GM18091	Cell line	HuVar	140,749,311	3,297	96.1	No	Yes
GM18507	Cell line	HapMap	116,001,927	2,717	99.2	Yes	Yes
GM18524	Cell line	HapMap	123,974,593	2,904	99.4	Yes	Yes
GM18526	Cell line	HapMap	68,506,615	1,605	98.2	Yes	Yes
GM18529	Cell line	HapMap	103,011,729	2,413	99.2	No	Yes
GM18532	Cell line	HapMap	93,010,560	2,178	98.9	Yes	Yes
GM18537	Cell line	HapMap	88,541,054	2,074	98.2	Yes	Yes
GM18540	Cell line	HapMap	107,018,419	2,507	99.3	Yes	Yes
GM18558	Cell line	HapMap	110,404,280	2,586	99.3	No	Yes
GM18561	Cell line	HapMap	94,941,108	2,224	98.7	No	Yes
GM18562	Cell line	HapMap	109,707,907	2,570	99.4	Yes	Yes
GM18563	Cell line	HapMap	132,909,807	3,113	95.9	No	Yes
GM18668	Cell line	Disease	142,395,245	3,335	99.4	Yes	Yes
GM18799	Cell line	Disease	117,599,230	2,754	99.2	Yes	Yes
GM18800	Cell line	Disease	109,551,224	2,566	99.2	Yes	Yes
GM18802	Cell line	Disease	87,204,605	2,042	98.8	Yes	Yes
GM18886	Cell line	Disease	138,604,386	3,246	95.1	Yes	Yes
GM18992	Cell line	HapMap	108,306,942	2,537	94.4	Yes	Yes

TABLE 6-continued

Sample ID	Sample Source	Sample Type	Sequence (raw bp)	Average Coverage	Percentage of Bases >= 50X	Reproducibility ?	Sanger Concordance ?
GM18995	Cell line	HapMap	96,468,405	2,259	99.5	No	Yes
GM18997	Cell line	HapMap	111,633,425	2,615	98.8	No	Yes
GM18998	Cell line	HapMap	99,785,735	2,337	99.4	Yes	Yes
GM18999	Cell line	HapMap	127,162,920	2,978	97.7	No	Yes
GM19000	Cell line	HapMap	66,999,861	1,569	98.0	Yes	Yes
GM19003	Cell line	HapMap	126,196,393	2,956	94.9	No	Yes
GM19005	Cell line	HapMap	143,461,749	3,360	96.3	No	Yes
GM19007	Cell line	HapMap	116,823,482	2,736	99.1	No	Yes
GM19012	Cell line	HapMap	121,510,893	2,846	99.8	Yes	Yes
GM19093	Cell line	HapMap	104,709,693	2,452	95.0	Yes	Yes
GM19099	Cell line	HapMap	108,885,873	2,550	98.2	Yes	Yes
GM19101	Cell line	HapMap	120,459,303	2,821	99.4	Yes	Yes
GM19116	Cell line	HapMap	71,500,299	1,675	99.2	No	Yes
GM19127	Cell line	HapMap	119,050,421	2,788	99.2	Yes	Yes
GM19130	Cell line	HapMap	66,366,273	1,554	96.9	No	Yes
GM19137	Cell line	HapMap	97,725,686	2,289	98.9	Yes	Yes
GM19141	Cell line	HapMap	110,866,363	2,597	99.1	Yes	Yes
GM19144	Cell line	HapMap	117,906,143	2,762	99.2	No	Yes
GM19152	Cell line	HapMap	84,729,187	1,984	99.2	Yes	Yes
GM19159	Cell line	HapMap	90,111,210	2,111	99.1	Yes	Yes
GM19172	Cell line	HapMap	74,654,792	1,749	96.6	No	Yes
GM19192	Cell line	HapMap	127,763,780	2,992	99.8	Yes	Yes
GM19200	Cell line	HapMap	114,675,886	2,686	99.7	Yes	Yes
GM19203	Cell line	HapMap	117,546,446	2,753	98.8	Yes	Yes
GM19207	Cell line	HapMap	84,803,031	1,986	98.7	Yes	Yes
GM19209	Cell line	HapMap	59,249,941	1,388	97.0	Yes	Yes
GM19223	Cell line	HapMap	70,582,882	1,653	98.0	Yes	Yes
GM19240	Cell line	HapMap	74,942,748	1,755	99.0	Yes	Yes
GM19776	Cell line	HapMap	91,506,428	2,143	93.3	No	Yes
GM19780	Cell line	HapMap	93,214,221	2,183	94.4	No	Yes
GM19782	Cell line	HapMap	147,554,997	3,456	97.0	No	Yes
GM19789	Cell line	HapMap	99,895,304	2,340	95.9	Yes	Yes
GM19794	Cell line	HapMap	112,615,959	2,638	95.6	Yes	Yes
GM20281	Cell line	HapMap	118,388,590	2,773	94.9	Yes	Yes
GM20332	Cell line	HapMap	134,954,116	3,161	94.4	Yes	Yes
GM20335	Cell line	HapMap	109,399,561	2,562	92.8	Yes	Yes
GM20341	Cell line	HapMap	96,681,315	2,264	92.5	Yes	Yes
GM20342	Cell line	HapMap	149,562,210	3,503	98.3	Yes	Yes

TABLE 6-continued

Sample ID	Sample Source	Sample Type	Sequence (raw bp)	Average Coverage	Percentage of Bases >= 50X	Reproducibility ?	Sanger Concordance ?
GM20344	Cell line	HapMap	112,547,107	2,636	97.6	Yes	Yes
GM20349	Cell line	HapMap	96,682,870	2,264	92.7	No	Yes
GM20357	Cell line	HapMap	128,110,988	3,001	95.9	No	Yes
GM20360	Cell line	HapMap	105,976,911	2,482	92.2	No	Yes
GM20363	Cell line	HapMap	114,582,012	2,684	94.9	No	Yes
GM20737	Cell line	Disease	86,947,571	2,036	98.4	Yes	Yes
GM20741	Cell line	Disease	131,676,642	3,084	99.5	Yes	Yes
GM20745	Cell line	Disease	120,425,678	2,821	99.4	Yes	Yes
GM20845	Cell line	HapMap	88,592,183	2,075	98.5	No	Yes
GM20846	Cell line	HapMap	94,474,722	2,213	98.9	No	Yes
GM20847	Cell line	HapMap	132,183,920	3,096	94.6	No	Yes
GM20849	Cell line	HapMap	94,859,450	2,222	99.2	Yes	Yes
GM20850	Cell line	HapMap	89,746,969	2,102	98.9	Yes	Yes
GM20851	Cell line	HapMap	105,058,248	2,461	98.9	No	Yes
GM20852	Cell line	HapMap	103,469,223	2,423	99.2	Yes	Yes
GM20853	Cell line	HapMap	67,451,488	1,580	99.0	Yes	Yes
GM20854	Cell line	HapMap	125,360,575	2,936	95.9	No	Yes
GM20856	Cell line	HapMap	125,206,711	2,933	95.1	Yes	Yes
GM20858	Cell line	HapMap	102,707,143	2,406	99.3	Yes	Yes
GM20859	Cell line	HapMap	107,012,009	2,506	98.9	No	Yes
GM20861	Cell line	HapMap	146,690,573	3,436	96.3	No	Yes
GM20862	Cell line	HapMap	121,310,107	2,841	99.4	Yes	Yes
GM20866	Cell line	HapMap	106,527,164	2,495	99.4	Yes	Yes
GM20869	Cell line	HapMap	88,099,219	2,063	99.4	Yes	Yes
GM20870	Cell line	HapMap	84,570,991	1,981	99.0	Yes	Yes
GM20871	Cell line	HapMap	104,048,645	2,437	98.9	No	Yes
GM20872	Cell line	HapMap	90,867,460	2,128	99.0	No	Yes
GM20873	Cell line	HapMap	108,700,925	2,546	99.3	No	Yes
GM20924	Cell line	Disease	120,376,414	2,819	99.2	Yes	Yes
GM21080	Cell line	Disease	66,554,012	1,559	97.3	Yes	Yes
blood01	Blood	N/A	67,892,054	1,594	99.9	No	No
blood02	Blood	N/A	75,235,946	1,766	99.9	No	No
blood03	Blood	N/A	71,324,606	1,674	99.9	No	No
blood04	Blood	N/A	58,883,762	1,382	99.9	No	No
blood05	Blood	N/A	74,862,133	1,757	99.9	No	No
blood06	Blood	N/A	77,267,380	1,814	99.9	No	No
blood07	Blood	N/A	55,719,056	1,308	99.9	No	No
blood08	Blood	N/A	64,495,882	1,514	99.9	No	No
blood09	Blood	N/A	67,663,353	1,588	99.9	No	No
blood10	Blood	N/A	57,362,443	1,347	99.9	No	No
blood11	Blood	N/A	53,823,416	1,264	99.9	No	No
blood12	Blood	N/A	73,097,398	1,716	99.9	No	No
blood13	Blood	N/A	73,858,165	1,734	99.9	No	No
blood14	Blood	N/A	87,675,439	2,058	99.9	No	No
blood15	Blood	N/A	74,484,474	1,749	99.8	No	No
blood16	Blood	N/A	59,096,764	1,387	99.8	No	No

TABLE 6-continued

Sample ID	Sample Source	Sample Type	Sequence (raw bp)	Average Coverage	Percentage of Bases >= 50X	Reproducibility ?	Sanger Concordance ?
blood17	Blood	N/A	65,114,672	1,529	99.9	No	No
blood18	Blood	N/A	41,759,247	980	99.9	No	No
blood19	Blood	N/A	71,949,103	1,689	99.9	No	No
blood20	Blood	N/A	81,225,381	1,907	99.9	No	No
blood21	Blood	N/A	70,214,097	1,648	99.9	No	No
blood22	Blood	N/A	72,674,504	1,706	99.9	No	No
blood23	Blood	N/A	74,340,749	1,745	99.9	No	No
blood24	Blood	N/A	64,015,737	1,503	99.9	No	No
blood25	Blood	N/A	73,147,784	1,717	99.8	No	No
blood26	Blood	N/A	41,950,444	985	99.8	No	No
blood27	Blood	N/A	62,771,860	1,474	99.8	No	No
blood28	Blood	N/A	47,085,570	1,105	99.8	No	No
blood29	Blood	N/A	74,840,986	1,757	99.9	No	No
blood30	Blood	N/A	73,612,767	1,728	99.9	No	No
blood31	Blood	N/A	70,446,967	1,654	99.9	No	No
blood32	Blood	N/A	86,513,773	2,031	99.9	No	No
blood33	Blood	N/A	78,330,087	1,839	99.9	No	No
blood34	Blood	N/A	76,890,117	1,805	99.9	No	No
blood35	Blood	N/A	63,472,751	1,490	99.9	No	No
blood36	Blood	N/A	77,259,799	1,814	99.9	No	No
blood37	Blood	N/A	74,384,590	1,746	99.9	No	No
blood38	Blood	N/A	87,075,653	2,044	99.9	No	No
blood39	Blood	N/A	61,490,312	1,444	99.9	No	No
blood40	Blood	N/A	83,490,415	1,960	99.9	No	No
blood41	Blood	N/A	94,474,694	2,218	99.9	No	No
blood42	Blood	N/A	79,180,999	1,859	99.9	No	No
blood43	Blood	N/A	70,106,334	1,646	99.9	No	No
blood44	Blood	N/A	66,239,225	1,555	99.9	No	No
blood45	Blood	N/A	76,565,215	1,797	99.8	No	No
blood46	Blood	N/A	66,932,062	1,571	99.9	No	No
blood47	Blood	N/A	37,972,652	891	99.8	No	No
blood48	Blood	N/A	66,880,850	1,570	99.9	No	No
blood49	Blood	N/A	65,267,319	1,532	99.9	No	No
blood50	Blood	N/A	63,720,579	1,496	99.9	No	No
blood51	Blood	N/A	64,485,398	1,514	99.9	No	No
blood52	Blood	N/A	90,657,228	2,128	99.9	No	No
blood53	Blood	N/A	83,058,297	1,950	99.9	No	No
blood54	Blood	N/A	86,145,665	2,022	99.9	No	No
blood55	Blood	N/A	77,159,945	1,811	99.9	No	No
blood56	Blood	N/A	88,169,014	2,070	99.9	No	No
blood57	Blood	N/A	60,859,847	1,429	99.9	No	No
blood58	Blood	N/A	72,504,883	1,702	99.9	No	No
blood59	Blood	N/A	83,924,327	1,970	99.9	No	No

The DNA extraction protocol used for our blood samples concluded with an overnight incubation at 65° C. in a Tris-based buffer. Subsequent experiments showed that this step reduced the mean size of the purified DNA; shearing was likely caused by acid hydrolysis during a temperature-induced pH shift of the buffer. Lower molecular mass genomic DNA is more readily denatured, and therefore more accessible to molecular inversion probes, which improves capture reaction performance. Consistent with this hypothesis, overnight incubation temperature lowered to 25° C. significantly reduces the percentage of target bases that yield high confidence genotype calls.

To assess reproducibility, a subset of 126 samples derived from cell line DNA (Appendix A) was processed twice, each time by a different operator on different liquid handling equipment. At least 92% of bases were called at >=50× coverage in all samples, with high agreement between replicates (Pearson correlation coefficient 0.868). Out of 5,177,206 total genotype calls compared, 17 were discordant, for a concordance rate of 0.999997. These occurred at only 5 unique genomic positions, consistent with systematic sequencing error as the primary cause.

Sanger Concordance

To assess the overall accuracy of our NGS genotype calls, the genotype calls from the NGS pipeline were compared to those generated by automated analysis (Mutation Surveyor, MS) of bi-directional Sanger sequence of PCR amplicons in a subset of 194 samples. Within a total of 6,997,906 bp of sequence called by both methods, 3,973 concordant and 1,220 discordant single nucleotide variant (SNV) genotype calls were observed. Through manual inspection of the Sanger trace(s) corresponding to discordant genotype calls, it was determined that 1,139 were MS errors, generally caused by low quality traces or misalignment of traces to reference. Supporting the conclusion that the majority of discordant calls corresponded to incorrect Sanger calls, the Ti/Tv ratio of concordant genotype calls was observed as 3.19, and 0.61 for discordant Sanger calls eliminated as MS errors. The remaining 81 discordant genotype calls that could not be resolved because the corresponding traces were ambiguous, were re-amplified and re-sequenced. For 71 of these calls, this process yielded new Sanger data that led to the conclusion that the original automated Sanger calls were incorrect. An additional discordant call was resolved by another approach as a NGS true negative (FIGS. **15**A-B), leaving 9 high-confidence discordant SNV calls (Table 7), corresponding to 8 NGS false positives and 1 NGS false negative. Table 7 shows a comparison of NGS genotype calls (alignment-only algorithm) to Sanger-derived Mutation Surveyor genotype calls. Sanger genotype calls were considered truth. TP, true positive calls (non-reference NGS, non-reference Sanger); FP, false positive calls (non-reference NGS, reference Sanger); FN, false negative calls (reference NGS, non-reference Sanger); TN, true negative calls (reference NGS, reference Sanger). dbSNP membership determined relative to version 129. Indel calls were considered unique if they differed by sequence pattern or equivalence region. Known indels are disease-causing mutations present in previously-annotated samples.

TABLE 7

			TP	FP	FN	TN
SNV	Heterozygous	dbSNP	2,495	0	1	6,992,746
		not dbSNP	247	8	0	
	Homozygous	dbSNP	1,245	0	0	
		not dbSNP	13	0	0	
	Unique		231	3	1	
Indel	Total		61	396	3	6,992,358
	Unique		17	27	2	
	Known		31	—	0	

The NGS SNV false positive rate was 1.14x10-6 (95% Wilson binomial confidence interval [5.80x10-7 2.26x10-6]). The false positive calls occurred at 5 unique genomic loci, 3 of which were at adjacent positions in a single exon of gene MCOLN1 due to realignment within GATK.

FIG. **16**A-D shows GM18540 is an aneuploid cell line and hence yields skewed allelic fractions. FIG. **16**A gives an IGV view of NGS data from GM18540 for the genotype call of interest (shown between vertical lines). FIG. **16**B shows bi-directional Sanger data for the variant-containing region. FIG. **16**C provides a histogram of allele ratios for all non-reference genotype calls in chromosome 11 derived from whole-genome shotgun sequencing (WGSS) of GM18540 and control sample GM18537. FIG. **16**D shows genome-wide relative coverage for GM18540. WGSS coverage data for each of the autosomes was binned into 50 Kb intervals and the log-ratio of the per-sample mean normalized values was plotted versus chromosome position. Dashed vertical lines denote chromosome boundaries; within a chromosome the ratios are arranged according to genomic position.

The NGS SNV false negative rate was 2.50x10-4 (95% Wilson binomial confidence interval [1.28x10-5 1.41x10-3]). The false negative call observed occurred in chromosome 11 of a sample previously characterized as aneuploid. Out of 473 NGS reads covering the false negative locus, 9.5% supported the correct heterozygous A/C genotype call (FIG. **16**A), with Sanger sequencing showing low peak height for the alternate A allele (FIG. **16**B). Shotgun full-genome sequencing of this sample demonstrated a bimodal distribution of allele ratios for heterozygous calls in chromosome 11 (FIG. **16**C), and illustrated variable chromosome copy numbers (FIG. **16**D), supporting the conclusion that this sample was aneuploid.

For indels, a total of 61 true positives, 394 false positives (27 unique alleles) and 3 false negatives (2 unique alleles, both in exon 1 of SMPD1) were observed. Of 31 clinically-relevant disease mutations, all 31 were detected.

iii. Detection of Pathogenic Mutations

The ability to detect variants that cause the Mendelian diseases targeted by the panel (Table 5) in the set of 194 cell line-derived samples was assessed. 55 of these samples were derived from individuals who were either carriers of or affected by one of the diseases being assayed and collectively contained a total of 95 previously-characterized disease mutations. During the design of our NGS workflow, we determined that three of these lesions would be inaccessible by our approach—two were large deletions spanning multiple exons, and one was contained within a region of paralogous sequence in the tenth exon of CFTR (Table 8). Of the 92 mutations we could expect to detect by NGS, we detected all 92 (Table 8). We also identified truncating (and likely disease-causing) mutations in two affected samples where previously only one mutation was known (FIGS. **17**A-D, Table 8), as well as 9 carriers in the set of 139 previously-uncharacterized HapMap, Thousand Genomes Project, and Human Diversity Panel samples (Table 8).

Table 8 shows pathogenic mutations detected in cell line-derived samples. Mutations underlined were determined a priori to be inaccessible by NGS and therefore not evaluated here. Mutations listed with an asterisk represent mutations in affected individuals that were previously unknown. Mutations listed in square brackets were present in Hapmap samples previously unannotated with respect to carrier status.

TABLE 8

Sample	Gene	Mut1 Common Name	Mut1 Found?	Mut2 Common Name	
GM04268	ASPA	E285A	Yes	E285A	Yes
GM00649	BCKDHA	Y438N	Yes	8 bp del exon 7	Yes
GM00650	BCKDHA	Y438N	Yes	—	Yes
GM01531	CFTR	PHE508DEL	Yes	PHE508DEL	Yes
GM02828	CFTR	V520F	Yes	PHE508DEL	Yes
GM04330	CFTR	1812 − IG > A	Yes	444delA	Yes
GM06966	CFTR	E92X	Yes	PHE508DEL	Yes
GM07381	CFTR	IVS19DS, +10 KB, C > T (3849 + 10 kb C > T)	Yes	PHE508DEL	Yes
GM07441	CFTR	621 + 1G > T	Yes	IVS16, G > A, +1(3120 + 1G > A)	Yes
GM07552	CFTR	ARG553TER	Yes	PHE508DEL	Yes
GM07732	CFTR	E60X	Yes	PHE508DEL	Yes
GM07857	CFTR	M11O1K	Yes	M11O1K	Yes
GM08338	CFTR	GLY551ASP	Yes	—	Yes
GM11275	CFTR	1-BP DEL, 3659C	Yes	PHE508DEL	Yes
GM11277	CFTR	ILE507DEL	Yes	ILE507DEL	Yes
GM11278	CFTR	Q493X	Yes	PHE508DEL	Yes
GM11280	CFTR	621 + 1G > T	Yes	711 + 1G > T	Yes
GM11281	CFTR	621 + 1G > T	Yes	PHE508DEL	Yes
GM11282	CFTR	621 + 1G > T	Yes	GLY85GLU	Yes
GM11283	CFTR	ALA455GLU	N/A	PHE508DEL	Yes
GM11284	CFTR	ARG560THR	Yes	PHE508DEL	Yes
GM11285	CFTR	Y1092X	Yes	PHE508DEL	Yes
GM11287	CFTR	P574H	Yes	PHE508DEL	Yes
GM11288	CFTR	G178R	Yes	PHE508DEL	Yes
GM11370	CFTR	444delA	Yes	1VS11 − 1G > A	Yes
GM11472	CFTR	ASN1303LYS	Yes	GLY1349ASP	Yes
GM11496	CFTR	GLY542TER	Yes	GLY542TER	Yes
GM11497	CFTR	GLY542TER	Yes	—	—
GM11723	CFTR	TRP1282TER	Yes	—	—
GM11859	CFTR	2789 + 5G > A	Yes	2789 + 5G > A	
G M11860	CFTR	IVS19DS, +10 KB, C > T(3849 + 10 kb C > T)	Yes	IVS19DS, +10 KB, C > T(3849 + 10 kb C > T)	
G M12444	CFTR	IVS10AS, G > A, −1 (1717 − IG > A)	Yes	—	—
G M12585	CFTR	ARG1162TER	Yes	—	—
G M12785	CFTR	ARG347PRO	Yes	GLY551ASP	Yes
GM12960	CFTR	ARG334TRP	Yes	c.3368 − 2A > T*	Yes

TABLE 8-continued

Sample	Gene	Mut1 Common Name	Mut1 Found?	Mut2 Common Name	
GM13423	CFTR	G85E	Yes	D1152H	Yes
G M13591	CFTR	ARG117HIS	Yes	PHE508DEL	Yes
GM18668	CFTR	CFTdele2, 3	N/A	PHE508DEL	Yes
GM18799	CFTR	2183delA		PHE508DEL	Yes
GM18800	CFTR	1898 + 1 G > A		PHE508DEL	Yes
GM18802	CFTR	Y122X		R1158X*	Yes
GM18886	CFTR	2143delT		PHE508DEL	Yes
GM20737	CFTR	R347H		—	—
GM20741	CFTR	3876delA		—	—
GM20745	CFTR	S549N		—	—
GM20924	CFTR	R75X		—	—
GM21080	CFTR	349delTT		—	—
GM11468	G6PC	R83C		Q347X	Yes
GM00502	HEXA	1278insTATC		1421 + 1G > C	Yes
GM03461	HEXA	1421 + 1G > C		G269S	Yes
GM05042	IKBKAP	2507 + 6T > C		2507 + 6T > C	Yes
GM02533	MCOLN1	IVS3 − 2A > G	Yes	del ex1-ex7	N/A
GM03252	SMPD1	L302P		—	—
GM13205	SMPD1	fsP330		—	—
G M16193	SMPD1	R496L		Arg608DEL	Yes
G M19116	CFTR	[ARG334TRP]	Yes	—	—
G M17363	IKBKAP	[2507 + 6T > C]	Yes	—	—
G M17366	IKBKAP	[2507 + 6T > C]	Yes	—	—
GM17365	IKBKAP	[2507 + 6T > C]	Yes	—	—
GM17364	IKBKAP	[2507 + 6T > C]	Yes	—	—
GM17360	MCOLN1	[IVS3 − 2A > G]	Yes	—	—
GM17362	HEXA	[1278insTATC]	Yes	—	—
GM18015	HEXA	[c.739C > T]	Yes	—	—
GM17362	HEXA	[G269S]	Yes	—	—

Genotyping by Assembly—Templated Alignment

Although substitutions comprise the majority of coding variation in the human genome, insertions and deletions (indels) are often clinically relevant. Indels, especially when large or present in cis with substitutions, are notoriously difficult to detect with short NGS reads. Assembly of short reads can improve indel detection sensitivity, but this is often at the cost of decreased SNV and indel specificity due to the presence of spurious contiguous sequence (contigs). An algorithm was devised termed Genotyping by Assembly-Templated Alignment (GATA), that first forms an assembly from reads partitioned into subsets by targeting arm sequence, then performs base quality- and coverage-informed genotyping by alignment of raw reads back to the assembled contigs (FIGS. **18**A-**18**E).

FIGS. **18**A-E depicts the next-generation DNA sequencing workflow. Genomic DNA samples are input to a molecular inversion probe capture reaction. Each target (depicted as grey and black regions) is captured by multiple probes that anneal to non-overlapping genomic intervals. PCR is performed using primers containing patient-specific barcodes, yielding barcode libraries. Equal volumes of the libraries are pooled and enter Illumina's Hiseq high-throughput sequencing workflow as shown in FIG. **18**B. Following sequencing, reads enter either the alignment only (AO, left) as depicted in FIG. **18**C or Genotyping by Assembly-Templated Alignment (GATA, right) analysis pipeline as depicted in FIG. **18**D. As shown in FIG. **18**C, AO first partitions reads by sample molecular barcode, then in parallel for all samples performs short read alignment, base quality recalibration, realignment around putative indels, and genotyping. As shown in FIGS. **18**D-E, GATA partitions reads first by sample molecular barcode, then by target. Reads are assembled into contigs that are then aligned to the reference genome. Raw reads are then aligned to the contigs, and raw read mapping and variant information relative to the reference is determined using reference-contig and read-contig alignments. Finally, base quality score recalibration and genotyping are performed on the mapped, raw reads.

The performance of GATA for indel genotyping was compared to the more conventional genotyping-by-alignment only (AO) algorithm used in the Sanger concordance studies. Across a set of 147 samples analyzed, both indel sensitivity and specificity were increased with GATA relative to AO (Table 9). GATA detected 23 unique insertions and deletions, which were confirmed by manual review of Sanger traces. Of these, 9 (39%) were not detected by AO in one or more samples, including BLM c.2207_2212delinsTAGATTC—the most common disease-causing mutation for Bloom syndrome in people of Ashkenazi Jewish descent—as well as several alleles in SMPD1 (Table 10), the gene associated with Niemann-Pick disease (FIGS. **19**A-D). Performance for substitutions was identical for both detection methods (AO and GATA).

Table 9 shows genotyping by assembly-templated alignment (GATA) improves detection of insertions and deletions. Raw variant alleles (positive calls) from 147 samples were filtered by depth and strand bias and categorized according to NGS data analysis method, alignment only (AO) or GATA. Calls were classified with GATA considered truth as true positive (TP), false positive (FP), and false negative (FN). Discordant calls, in all cases, were confirmed by manual review of corresponding Sanger traces and found to be GATA TP or TN, rather than FP or FN. Variant calls flagged as low-confidence are considered uncalled. Polymorphisms in the first exon of SMPD1 accounted for the majority of uncalled and discordant alleles, which were not considered in accuracy calculations.

TABLE 9

	O	ATA
TP	04	11
FP	8	
FN	7	
uncalled*	0	0
sensitivity	.696	.0
precision	.786	.0

Table 10 shows the frequency distribution of variant genotypes for the STR at SMPD1 exon 1 representing various combinations of (i) the minor reference allele (0), (ii) a substitution (snp), (iii) insertions (+6 and +12 bp in length), and (iv) deletions (−6, −12, and −18 bp in length) as determined by GATA and confirmed by manual inspection of Sanger traces.

TABLE 10

Genotype	Frequency
snp/snp	42
−6/snp	41
−6/−6	15
−12/0	8
snp/0	8
−6/0	7
−6/−12	7
−12/snp	4
−18/0	3
+12/snp	2
+6/snp	2

TABLE 10-continued

Genotype	Frequency
+6/0	1
−12/−12	1

As seen in FIGS. **19**A-D, GATA correctly genotypes insertions and deletions that are undetectable by the Alignment Only method. Read from top to bottom, each figure provides tracks for cumulative depth of coverage (vertical grey bars); representative MIP alignments (horizontal grey bars) with mismatches (letters), and gaps (dashed lines); chromatogram; reference DNA and amino acid sequence for FIG. **19**A heterozygous BLM c.2207_2212delinsTAGATTC in sample GM04408 as well as several alleles in the first exon of SMPD1 including FIG. **19**B a heterozygous 18 bp deletion in sample GM20342 (minus strand), FIG. **19**C a heterozygous 12 bp insertion and homozygous substitution in sample GM1 7282 (plus strand), and FIG. **19**D compound heterozygous 6 and 12 bp deletions in sample GM00502 (minus strand). Chromatogram trace offsets corresponding to specific heterozygous insertion and deletion patterns are indicated with slanted lines color coded by reference base. For clarity offsets are shown for FIG. **19**C and FIG. **19**D only.

Simulation to Assess Detectability of Rare Pathogenic Mutations

While detectability for all disease-causing mutations present in the sample set was empirically demonstrated, there exist a number of disease-causing mutations for which samples cannot be readily obtained. To assess whether the NGS workflow can detect these additional mutations, the stimulations were performed in silico. Since detectability can be affected by any element of the workflow, a simulator was implemented that employed read sets from actual samples rather than model reads derived from the reference genome at uniform coverage. This allowed for realistic representation of target abundance distribution, neighboring in cis variants, as well as cycle- and context-dependent sequencing errors. Disease-causing variants were introduced into raw reads by a Bernoulli process, with an average 0.5 probability of introducing the lesion, to simulate the heterozygous genotypes carrier screening aims to detect.

A total of 81 heterozygous variants were simulated in a read set of at least 144 samples with the exception of c.1521_1523delCTT (F508del), the most common disease-causing mutation for cystic fibrosis in Caucasian populations, as shown in Table 11. This mutation was present in several samples, which were removed from simulation analysis (Materials and Methods). Of the simulated variants 67 (83%) were correctly genotyped in all (generally 145/145) samples and only four relatively large (>7 bp) deletions were undetected in one or more samples. High-confidence genotype calls were not made for the remaining 10 variants. No variants were found to be undetectable in all samples. Table 11 gives the performance results of GATA for detecting clinically-relevant mutations by simulation.

TABLE 11

Variant	Samples Simulated	Variant Positive	Variant Uncalled	Variant Negative
BLM c.2207_2212delinsTAGATTC	146	146	0	0
CFTR c.1923_1931delCTCAAAACTinsA	147	147	0	0
CFTR c.1973_1985del13insAGAAA	146	146	0	0
CFTR c.723_743 + 1delGAGAATGATGATGAAGTACAGG (SEQ ID NO: 6325)	147	147	0	0
CFTR c.3067_3072delATAGTG	147	147	0	0
CFTR_c.650_659delAGTTGTTACA (SEQ ID NO: 6326)	145	145	0	0
CFTR_c.1871_1878delGCTATTTT	145	145	0	0
CFTR_c.739_742dupTACA	145	145	0	0
CFTR_c.578_579 + 5delAAGTATG	145	145	0	0
CFTR_c.3421_3424dupAGTA	145	145	0	0
BLM_c.991_995del5	145	145	0	0
CFTR_c.2589_2599delAATTTGGTGCT (SEQ ID NO: 6327)	145	46	7	92
CFTR_c.3664_3665insTCAA	145	145	0	0
CFTR_c.2634_2641delGGTTGTGC	145	143	1	1
CFTR_c.156_163dupATTGGAAA	145	145	0	0
CFTR_c.522_526delAATAA	145	145	0	0
ABCC8_c.259_268del10	145	141	3	1
CFTR_c.1616_1617dupTA	145	145	0	0

TABLE 11-continued

Variant	Samples Simulated	Variant Positive	Variant Uncalled	Variant Negative
CFTR_c.3068_3072delTAGTG	145	145	0	0
FANCC_c.356_360del5	145	145	0	0
CFTR_c.861_865delCTTAA	145	145	0	0
ABCC8_c.2835_2838delGAGA	145	145	0	0
CFTR_c.319_326delGCTTCCTA	145	145	0	0
CFTR_c.2249_2256del8	145	145	0	0
CFTR_c.1792_1798delAAAACTA	145	145	0	0
CFTR_c.2241_2248delGATACTGC	145	145	0	0
G6PC_c.462_466deITTTGT	145	145	0	0
CFTR_c.35_36insTATCA	145	145	0	0
HEXA_c.1471_1475delTCTGA	145	145	0	0
PCDH15_c.996_999delGGAT	145	145	0	0
ASPA_c.568_574del7	145	144	0	1
CFTR_c.3184_3188dupCTATG	145	145	0	0
SMPD1_c.1657_1663delACCGCCT	145	145	0	0
CFTR_c.1162_1168delACGACTA	145	145	0	0
BCKDHB_c.163_166dupACTT	145	145	0	0
BCKDHA_c.861_868delAGGCCCCG	145	145	0	0
CFTR_c.3773dupT	145	145	0	0
CFTR_c.1155_1156dupTA	145	145	0	0
CFTR_c.3889dupT	145	145	0	0
HEXA_c.1274_1277dupTATC	145	145	0	0
CFTR_c.262_263delTT	244	144	0	0
CFTR_c.326_327delAT	245	145	0	0
CFTR_c.3691delT	145	145	0	0
CFTR_c.3528delC	144	144	0	0
BLM_c.2407dupT	145	145	0	0
CFTR_c.1521_1523delCTT	131	131	0	0
HEXA_c.915_917delCTT	145	145	0	0
G6PC_c.379_380dupTA	145	145	0	0
CFTR_c.2012delT	144	144	0	0
SMPD1_c.1829_1831delGCC	144	144	0	0
CFTR_c.1029delC	145	127	18	0
CFTR_c.2737_2738insG	145	145	0	0
CFTR_c.2947_2948delTT	145	142	3	0
CFTR_c.1911delG	145	145	0	0
CFTR_c.803delA	145	145	0	0
CFTR_c.1519_1521delATC	145	145	0	0
CFTR_c.805_806delAT	145	18	127	0

TABLE 11-continued

Variant	Samples Simulated	Variant Positive	Variant Uncalled	Variant Negative
CFTR_c.2215delG	145	137	8	0
FANCC_c.67delG	145	145	0	0
CFTR_c.935_937delTCT	145	145	0	0
CFTR_c.2175dupA	145	145	0	0
CFTR_c.3530delA	145	145	0	0
CFTR_c.531delT	145	145	0	0
CFTR_c.1021_1022dupTC	145	127	18	0
CFTR_c.3659delC	145	145	0	0
DLD_c.104dupA	144	144	0	0
CFTR_c.2052dupA	144	144	0	0
CFTR_c.313delA	145	145	0	0
G6PC_c.79delC	145	145	0	0
CFTR_c.442delA	145	145	0	0
CFTR_c.1477_1478delCA	145	145	0	0
CFTR_c.1545_1546delTA	145	145	0	0
BCKDHA_c.117delC	145	145	0	0
CFTR_c.1418delG	145	145	0	0
CFTR_c.1976delA	145	145	0	0
CFTR_c.3536_3539delCCAA	145	145	0	0
CFTR_c.948delT	145	145	0	0
CFTR_c.2052delA	145	145	0	0
BCKDHB_c.595_596delAG	145	145	0	0
G6PC_c.980_982delTCT	145	145	0	0
CFTR_c.3039delC	145	145	0	0

DISCUSSION

Robustness, completeness, and accuracy are three of the main factors that define the utility of a genetic carrier testing workflow in a clinical laboratory. By utilizing a target enrichment methodology that is performed in a single tube and requires no mechanical shearing or purifications of individual samples, methods of the invention provide an automated NGS workflow that yields highly-reproducible results across samples and operators. This reproducibility ensures that samples will not have to be rerun frequently, minimizing both turnaround time and per-sample cost.

Because each clinically meaningful basepair must be sequenced before an actionable medical report can be generated, a high level of completeness minimizes the amount of costly re-work necessary for a sample. Methods of the invention demonstrate completeness that is consistent with low to no re-work for the samples studied, and substantially better than other previously-reported methods using multiplex target capture or PCR with NGS. This improvement is likely the result of a number of optimizations we have made relative to previous reports including the use of a tiling MIP design that ensures multiple probes capture each base and the use of a DNA extraction protocol that effortlessly shears the DNA to a lower molecular mass.

Regarding accuracy, the only observed SNV false negative was in a sample that exhibited skewed allele ratios along the chromosome, which should not commonly occur when testing for germline mutations in clinical specimens derived from whole blood. Additionally, the SNV false positive rate of approximately 1.1 per million basepairs corresponds to a low confirmation burden for clinical testing and surpasses values previously reported. Given the small target set and the rare nature of indels, it is difficult to give a precise measurement of our accuracy for indels in genera. However, this data suggests that the use of GATA substantially improves our ability to detect small lesions. Additionally, a sensitivity of 100% by both AO and GATA was observed across the set of disease-causing insertions and deletions in carrier and affected samples.

It is worth noting that measuring accuracy to a sufficient level of precision and generality can be challenging within conserved coding regions because selective pressure limits the spectrum of variation present. While a large number of samples were sequenced, the relatively small size of our target limited the number of unique alleles observable and meant that approximately 90% of such variants were common (i.e. present in dbSNP). Nonetheless, there is no a priori reason to believe that the measured accuracy will not generalize to other rare and private mutations present in the targeted loci. Supporting this point, these simulations using real data and controlled for sample-to-sample variability indicate that one can detect a number of very rare disease causing alleles of different types and sequence contexts, including insertions (up to 12 bp), deletions (up to 22 bp) and complex combinations thereof.

The reference standard one considers ground truth can impose a ceiling on measurable accuracy. Automated analysis of what is widely deemed the 'gold standard' for DNA sequencing was employed: bi-directional Sanger traces derived from PCR amplicons. FIGS. **20**A-**20**B shows NGS detects allele dropout in Sanger sequencing reactions. FIG. **20**A-**1**, FIG. **20**A-**2**, and FIG. **20**A-**3** show dropout of reference allele leads to homozygous non-reference call by Sanger sequencing, but heterozygous non-reference call by NGS, in BLM exon 12 of GM18034. Shown from top to bottom, (FIG. **20**A-**1**) original PCR primer pair: expected reference sequence trace, sample forward trace, sample reverse trace; (FIG. **20**A-**2**) re-designed PCR primer pair: expected reference sequence trace, sample forward trace, sample reverse trace; IGV of NGS data.

FIG. **20**B-**1**, FIG. **20**B-**2**, and FIG. **20**B-**3** shows dropout of non-reference allele leads to homozygous reference call by Sanger sequencing, but heterozygous non-reference call by NGS, in DLD exon 9 of sample GM11370. Shown from top to bottom, expected reference sequence trace, sample forward trace, sample reverse trace. Shown from top to bottom, (FIG. **20**B-**1**) original PCR primer pair: expected reference sequence trace, sample forward trace, sample reverse trace; (FIG. **20**B-**2**) re-designed PCR primer pair: expected reference sequence trace, sample forward trace, sample reverse trace; IGV of NGS data. Project genotyping data was employed, 12 NGS false negatives and 7 false positives would have been observed in the subset of samples characterized by this approach. Because these were all shown by Sanger analysis to be HapMap Project genotyping errors, this would have underestimated both sensitivity and specificity.

The NGS workflow detected allele dropout in the Sanger data, a known limitation of that technology (FIGS. **20**A-**1** through **20**B-**3**) and not surprising since each base sequenced by NGS was captured by multiple probes with independent targeting arms. Had the less laborious and more commonly-used reference of Hapmap Project genotyping data been employed, 12 NGS false negatives and 7 false positives would have been observed in the subset of samples characterized by this approach (Table 12). This would have underestimated both sensitivity and specificity.

Table 12 shows concordance of NGS genotypes with HapMap data. All NGS positions called with high confidence (minimum 50× coverage and strand bias <=0) that intersected Hapmap release 27 phase II+III genotyping data were evaluated, for a total of 5,337 genotypes across 83 samples. True negative: reference called by both NGS and HapMap; true positive: non-reference (heterozygous or homozygous) called by both NGS and HapMap; false positive: non-reference called by NGS, reference called by HapMap; false negative: reference called by NGS, non-reference called by HapMap. Specificity: TN/(TN+FP); sensitivity: TP/(TP+FN).

TABLE 12

True negatives	4,233
True positives	1,085
False positives	7
False negatives	12
Specificity	0.998
Sensitivity	0.989

Indel detection methods that only employ gapped alignment of short reads to reference are often limited by false positives introduced by systematic, context-dependent sequencing error, and false negatives introduced by failure of the aligner to open or extend gaps. An assembly-based paradigm would address these limitations but raw contigs do not always carry base quality and coverage information. The GATA algorithm combines these approaches to deliver sensitive and specific indel detection with SNV performance on par with a traditional alignment-only pipeline.

Many alleles detected exclusively by GATA were from a short tandem repeat (STR) region encoding the N-terminal signal peptide in SMPD1 (Table 10). Consistent with previous reports, GATA detected non-reference alleles in 96% of samples, a rate that is strikingly high because hg18 contains a minor allele that is frequently substituted (V36A). While common hexanucleotide indels at this locus are clinically benign, any pathogenic mutation present in cis would likely be missed using a conventional approach for variant detection. Indeed, when reads were aligned independently, several genomic positions in this region consistently fell below our specified coverage threshold. GATA therefore should yield higher sensitivity for rare mutations linked to polymorphisms in the first exon of SPMD1 and potentially other STR loci as well.

The simulation methodology applied here attempts to assess detectability of rare pathogenic mutations in a highly realistic manner. Simply deriving reads from a reference genome modified to include the mutation of interest can overestimate the detection probability because of real-world factors that would otherwise render the mutation undetectable. Additionally, we are able to determine whether a mutation is sometimes, rather than always or never, detectable because it is simulated in the read sets of hundreds of samples; e.g., this could occur in a particular genetic background with a low-frequency in cis variant that interferes with alignment of reads containing the mutation. Nonetheless, certain mutation types, in particular large deletions are still not amenable to this paradigm because they could fundamentally alter the distribution of reads generated across the relevant region. In these cases, either human samples or synthetic templates remain the only way to assess detectability.

In conclusion, an automated, integrated workflow that converts human genomic DNA isolated from blood or cell lines into clinically-relevant variant calls was presented by this example. High genotype concordance was achieved with conventional electrophoretic sequencing across a set of 15 genes. In addition, this example demonstrates the ability to detect a range of important disease-causing mutations. The pipeline analysis presented allows for sensitive and specific detection of indels, while simultaneously incorporating raw base quality and coverage into SNV genotype calls. Realistic simulation on actual run data indicates that a number of pathogenic mutations undetectable by a traditional alignment-based genotyping approach are accessible by GATA. Collectively, the data shows that this workflow has met three of the major requirements of a clinical carrier screening assay, supporting the notion that NGS is ready for clinical use.

It should be appreciated that the preceding examples are non-limiting and aspects of the invention may be implemented as described herein using alternative techniques and/or protocols that are available to one or ordinary skill in the art.

It will be clear that the methods may be practiced other than as particularly described in the foregoing description and examples. Numerous modifications and variations of the present disclosure are possible in light of the above teachings and, therefore, are within the scope of the claims. Preferred features of each aspect of the disclosure are as for each of the other aspects mutatis mutandis. The documents including patents, patent applications, journal articles, or other disclosures mentioned herein are hereby incorporated by reference in their entirety. In the event of conflict, the disclosure of present application controls, other than in the event of clear error.

Lengthy table referenced here

US12129514-20241029-T00001

Please refer to the end of the specification for access instructions.

Lengthy table referenced here

US12129514-20241029-T00002

Please refer to the end of the specification for access instructions.

LENGTHY TABLES

The patent contains a lengthy table section. A copy of the table is available in electronic form from the USPTO web site (https://seqdata.uspto.gov/?pageRequest=docDetail&DocID=US12129514B2). An electronic copy of the table will also be available from the USPTO upon request and payment of the fee set forth in 37 CFR 1.19(b)(3).

SEQUENCE LISTING

The patent contains a lengthy sequence listing. A copy of the sequence listing is available in electronic form from the USPTO web site (https://seqdata.uspto.gov/?pageRequest=docDetail&DocID=US12129514B2). An electronic copy of the sequence listing will also be available from the USPTO upon request and payment of the fee set forth in 37 CFR 1.19(b)(3).

What is claimed is:

1. A method for capturing and sequencing a nucleic acid target, the method comprising:

providing a sample comprising human nucleic acid;

introducing a probe set comprising a plurality of different molecular inversion probes, wherein each probe is designed to capture one of a plurality of overlapping sub-regions of a target on a strand of the nucleic acid;

hybridizing the probes to the strand;

converting the hybridized probes into covalently-closed circularized probes, thereby capturing the overlapping sub-regions of the target, wherein at least two different molecular inversion probes capture any given single base along the target;

isolating the circularized probes;

amplifying the isolated circularized probes to generate amplicons;

sequencing the amplicons to generate sequence reads of the captured sub-regions; and

analyzing the sequence reads to detect a mutation in said target nucleic acid specific for a disease.

2. The method of claim **1**, wherein the amplification step requires a single set of primers.

3. The method of claim **1**, wherein the disease is selected from the group consisting of: Familial hyperinsulinism, Canavan disease, Maple Syrup Urine disease, Bloom syndrome, Cystic fibrosis, Dihydrolipoamide dehydrogenase deficiency, Fanconi anemia, Glycogen Storage disease, Tay-Sachs diseases, Familial dysautonomia, Mucolipidosis, Usher syndrome, and Neimann-Pick disease.

4. The method of claim **1**, wherein the plurality of sub-regions comprises coding and non-coding regions of the human nucleic acid.

5. The method of claim **1**, wherein the probe set is a plurality of probe sets, each probe set specific for a different target nucleic acid and wherein each target nucleic acid is specific for a different disease.

6. The method of claim **1**, wherein said analyzing step comprises:

assembling the sequence reads into contigs; aligning the contigs to a reference; and

aligning the sequence reads to the contigs to determine mapping and variant information of the sequence reads relative to said reference to obtain a genotype at said mutation.

7. The method of claim **1**, wherein the overlapping sub-regions are at least 10 base-pairs in length.

8. The method of claim **1**, wherein the plurality of overlapping sub-regions of the target nucleic acid are from a gene selected from the list consisting of: ATP-binding cassette, sub-family C (CFTR/MRP), member 8 (ABCC8); aspartoacylase (ASPA); branched chain keto acid dehydrogenase E1, alpha polypeptide (BCKDHA); branched chain keto acid dehydrogenase E1, beta polypeptide (BCKDHB); Bloom Syndrome, RecQ Helicase-Like (BLM); cystic fibrosis transmembrane conductance regulator (CFTR), clarin 1 (CLRN1); dihydrolipoamide dehydrogenase (DLD); Fanconi anemia, complementation group C (FANCC); glucose-6-phosphatase, catalytic subunit (G6PC); hexosaminidase A alpha polypeptide (HEXA); kinase complex-associated protein (IKBKAP); mucolipin 1 (MCOLN1); protocadherin-related 15 (PCDH15); and sphingomyelin phosphodiesterase 1, acid lysosomal (SMPD1).

9. The method of claim **1**, wherein each probe is a molecular inversion probe (MIP) that comprises an oligonucleotide with

a first targeting arm at a 5' end and

a second targeting arm at a 3' end, wherein the first and second targeting arms are capable of specifically hybridizing to a first and second regions flanking one of the overlapping subregions.

10. The method of claim **9**, wherein each MIP includes a differentiator tag between the first and second targeting arms, wherein the differentiator tags are different across the probe set.

11. The method of claim **10**, wherein each differentiator tag provides a unique sequence tag to an individual target molecule captured by one of the plurality of different molecular inversion probes.

12. The method of claim **11**, wherein, after the amplifying and sequencing steps, the differentiator tags distinguish independent target molecules from one another.

13. The method of claim **11**, wherein the enzymatic removal uses an exonuclease.

14. The method of claim **9**, wherein the isolating step comprises enzymatic removal of non-circularized nucleic acid.

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